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Evaluation of IL-6, IL-10 and EG-VEGF at time of oocytes retrieval in correlation with the outcome of IVF-ICSI cycle

A Thesis

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By

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

وَيَسْـَٔلُونَكَ عَنِ ٱلرُّوحِۖ قُلِ ٱلرُّوحُ مِنْ أَمْرِ رَبِّى وَمَآ أُوتِيتُم مِّنَ ٱلْعِلْمِ إِلَّا قَلِيلا .

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Supervisor Certification

I certify that this thesis entitled (Evaluation of IL- 6, IL-10 and EG-VEGF at time of oocytes retrieval in correlation with the outcome of IVF-ICSI cycle) was prepared under my supervision at the College of Medicine/University of Karbala as a partial Fulfilment of the requirement for the degree of Master of Science in Medical Microbiology.

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Abstract

Infertility is defined as the inability to achieve pregnancy after 12 month or more of unprotected sexual intercourse. Ovulation disorders, such as Polycystic Ovary Syndrome (PCOS) and Poor Ovarian Reserve (POR), are among the female factors contributing to infertility. The ovarian tissue, along with immune cells, produces cytokines, which play a crucial role in follicular growth, steroidogenesis, leukocyte recruitment, and tissue remodeling during ovulation, luteinization, and luteolytic. This study aimed to evaluate the levels of Interleukin-6 (IL-6), Interleukin-10 (IL-10), and Endocrine Gland-Derived Vascular Endothelial Growth Factor (EG-VEGF) in follicular fluid and serum, and to investigate the correlation between these markers and the outcomes of Intracytoplasmic Sperm Injection (ICSI).

This cross-sectional study included subjects divided into four groups based on the cause of infertility: unexplained infertility, male factor, female factor, and combined factors. The study was conducted at Al-Kafeel Super specialty Hospital in Karbala from February 2024 to June 2024.

Serum and follicular fluid samples were collected on the day of oocyte retrieval, and the levels of IL-6, IL-10, and EG-VEGF were measured using the ELISA technique

The result of present study showed that FSH and LH were significantly higher in the female and combined factor groups compared to the male and unexplained factor groups, while E2 day2, AMH, and E2 on the day of injection HCG levels were lower in the female and combined factor groups compared to the male and unexplained factor groups. In addition, the total number of oocytes, fertilization rate, Embryo Grade I (GI), Embryo Grade II (GII), number of transferred embryos, and pregnancy rate in unexplained and male factor were high significantly different from the female and combined factor groups.

Regarding, the serum and follicular levels of cytokines, there were no significant differences were observed in the levels of IL-6, IL-10, and EGVEGF in follicular fluid and serum across the patient groups.

Furthermore, IL-6 levels in serum were significantly higher in pregnant women compared to non-pregnant women. In addition, EG-VEGF levels in follicular fluid showed a negative correlation with positive pregnancy outcomes.

Moreover, in this study there were positive correlations were observed between EG-VEGF in serum and Embryo Grade III (GIII), and between EG-VEGF in follicular fluid and Embryo Grade II (GII). In addition, Positive correlations were also found between IL-6 and EG-VEGF levels in serum and their corresponding levels in follicular fluid.

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List of Abbreviations

Code	Word
AMH	**Anti mullerian hormone**
ART	**Assisted Reproductive Technology**
ASRM	**American Society of Reproductive Medicine**

B-HCG	**Beta-Human Chronic Gonadotrpin**
COC	**Cumulus Oocyte Complex**
DOR	**Diminished Ovarian reserve**
E2	**Estradiol II**
ELISA	**Enzyme-Linked immunoSorbent Assay**
FF	**Follicular fluid**
FSH	**Follicle-stimulating hormone**
GI	**Grade 1**
GII	**Grade II**
GIII	**Grade III**
GV	**Germinal Vesicle**
ICSI	**Intracytoplasmic sperm injection**
IL	**Interleukin**
IVF	**In Vitro Fertilization**
LH	**Luteinizing hormone**
MI	**Metaphase 1**
MII	**Metaphase 1I**
ng/ml	**Nanogram per milleter**
ORT	**Ovarian Reserve Test**
PCOM	**Polycystic ovary morphology**
PCOS	**Polycystic ovary syndrome**
POR	**Poor Ovarian Reserve**

PVP	**Polyvinylpyrrolidone**
Pg/ ml	**Pikogram per milleter**
Rpm	**Rotation per minute**
WOI	**Window of implantion**
WHO	**World Health Organization**

Chapter One

Introduction

And

Literature Review

1.1 Introduction:

Infertility is a disease characterized by the failure to establish a clinical pregnancy after 12 months of regular and unprotected sexual intercourse (Vander Borght and Wyns 2018). The most common causes of infertility are Female factor 40% and male factor 30%.(El Adlani, Benksim et al. 2021). The prevalence of infertility continues to rise in the world for several reasons, including the age of conception and current lifestyle (Lemseffer, Terret et al. 2022).

Anovulation and abnormalities in the semen quality are the most prevalent reasons for infertility. Polycystic ovary syndrome (PCOS) is the most prevalent etiology of anovulatory infertility in women, accounting for around 40% of female infertility (Sharif 2022). It is an endocrinological problem that affects about 5%-10% of women of childbearing age all over the world. Oligoanovulation, clinical and/or biochemical hyperandrogenism, and polycystic ovary morphology (PCOM) on ultrasonography are the most important clinical manifestations of PCOS. It was reported that approximately 80% of anovulation infertility was caused by PCOS (Gao, Liu et al. 2023).

Regarding, Poor Ovarian reserve (POR). It is refers to the decline in fertility caused by the loss of normal ovarian function (Wang, Pan et al. 2023).

The Intracytoplasmic sperm injection (ICSI) is a micromanipulation technique that allows the injection of one spermatozoon into the ooplasm of a metaphase II oocyte. In humans, ICSI has been utilized for more than a quarter of a century and has become one of the most widely used assisted reproductive technologies for reproduction in humans. The widespread use of ICSI is the result of the capacity for this procedure to be utilized in

addressing many reproductive problems, such as lack of sperm motility and globozoospermia, which are mainly related to male infertility. Furthermore, ICSI has been utilized to overcome some problems when there is female infertility, such as less-than-optimal quality oocytes and/or when it is only possible to collect a small number of oocytes (Briski and Salamone 2022).

Cytokines can modulate the function of immune cells within the ovary in the context of reproduction. The ovarian interleukins, which are secreted by the granulosa cells and other immune cells within the ovaries and follicles along with hormonal changes, regulate various functions, including folliculogenesis, oogenesis, ovulation, fertilization, embryonic development, implantation, formation, and regression of the corpus luteum. One of the success factors of in vitro fertilization (IVF) is the quality of the obtained oocytes (Gavrilovic, Cekovic et al. 2022).

Moreover, Numerous cytokines were detected in follicular fluid, the role of which in reproductive physiology seems crucial. They influence the development and maturation of the follicle, ovulation, and corpus luteum formation, as well as embryo implantation and maintenance of pregnancy (Adamczak, Ukleja-Sokołowska et al. 2021).

IL-6 is a pleiotropic cytokine with multiple effects that can vary depending on the physiological environment. IL-6 promotes T cell population expansion, B cell differentiation, and proliferation of many cells it can also regulate the acute phase response and various homeostasis functions, including glucose metabolism, lipid metabolism, and insulin resistant (Gavrilovic, Cekovic et al. 2022). Significant difference in serum IL-6 levels between unexplained infertile and fertile women suggests that this cytokine may be involved in pathophysiology of unexplained infertility (Demir, Guven et al. 2009).

In addition, Endocrine gland-derived vascular endothelial growth factor (EG-VEGF), also termed as prokineticin (PK1), is the first protein identified as a selective angiogenic mitogen acting on the endothelial cells of steroidogenic endocrine glands. Serum concentrations of EG-VEGF were associated with the pregnancy outcome. So EG-VEGF may be applicable as an indicator for IVF pregnancy outcome, but more experimental supports such as EG-VEGF expression in endometrium during WOI phase are needed. Moreover, as serum concentrations of EG-VEGF may vary during the luteal phase, further study with sampling at multiple time-points will assure its role to predict the clinical pregnancy outcome (Gao, Zhao et al. 2012).

Regarding, Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that plays a crucial, and often essential, role in preventing inflammatory and autoimmune pathologies (Iyer and Cheng 2012).

The anti-inflammatory cytokines (serum IL-10) some study showed significant decrease in their levels in the infertile women when compared with the fertile women (Hassan, Khalil et al. 2022)

Aim of the Study

The aim of this study to investigate the usefulness of serum and follicular fluid levels of immunological markers (EG-VEGF, IL-6 and IL-10) in infertile females with different causes (male factor, female factor and unexplained infertility) as predicator for early ICSI outcomes; the following objective achieved this:

1- selection of women undergoing IVF.

2- completing the basic laboratory workup to confirm the diagnosis subfertility.

3- Collection of the demographic data and laboratory results in a questionnaire.

4- Collection of blood and follicular fluid sample from the female on the day of egg retrieval and injection and separation of serum samples.

5- Use of serum samples for measurement of IL-6, IL-10 and EG-VEGF

6- Monitoring the outcome of IVF by serum pregnancy test one appropriate.

1.2 Literature Review

1.2.1. Definition of infertility:

Infertility is a disease characterized by the failure to establish a pregnancy after 12 months of regular and unprotected sexual intercourse (Vander Borght and Wyns 2018). Sterility is a condition of involuntary childlessness. In contrast, infertility is a condition of having difficulty conceiving (Royfman, Shah et al. 2021).

1.2.2. Epidemiology of infertility:

Infertility is a big health problem worldwide as it has been estimated that in 2010 there are 48.5 million (45.0 million, 52.6 million) infertile couples worldwide. World Health Organization (WHO) defines infertility as "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse". Meanwhile the WHO's epidemiologic definition of infertility as "women of reproductive age at risk of becoming pregnant who report unsuccessfully trying for a pregnancy for more than two years (Elhussein, Ahmed et al. 2019).

Generally, (20-35%) of infertility cases are due to female cause, (20-30%) of cases are due to male cause, (25-40%) of cases due to both of male and female (combined) etiologies and 10% unexplained infertility (Mustafa, Sharifa et al. 2019) (El Adlani, Benksim et al. 2021).

In addition, the infertility rate is 10-15% for women younger than 35 years, this rate increases to about 33% for women in 35-40 age group, while 87% of 45 years old women have no possibility of conceiving a child, after 35 years of age. Infertility is present in 50% of couples, and the percentage of those with primary infertility (Al-Ani, Al-Kasser et al. 2021)

Moreover, Secondary infertility is the most common form of female infertility around the globe, often due to reproductive tract infections (Vander Borght and Wyns 2018)

1.2.3. Type of Infertility:

Infertility is classified as primary or secondary.

Primary infertility is denoted for those women who have not been conceived previously.

In secondary infertility, there is at least one conception but fails to repeat (Abebe, Afework et al. 2020). It infertility is the failure to conceive following a previous pregnancy in the absence of contraception, breastfeeding, or postpartum amenorrhea. Primary infertility is more typical than secondary infertility in affluent nations; however, the turnaround is valid in developing nations (Magdum, Chowdhury et al. 2022). Different risk factors are attributed to secondary infertility including lifestyle variables such as diet, obesity, drinking, smoking, and environmental hazards, as well as secondarily connected factors to human infertility such as childbirth complications, postpartum practices, and symptoms of sexually transmitted diseases. Other prevalent causes of female infertility include anovulatory disorders, polycystic ovarian syndrome, peri-tubovarian adhesions, endometriosis, and uterine and cervical factors (Fatima, Gilani et al. 2024).

1.2.4. Risk factors of infertility:

Among the risk factors for infertility are:

1. Age

A systematic analysis of infertility incidence in more than 190 countries and regions around the world showed that in 2010, women at the age of 20-44 years suffered from a primary infertility incidence of 1.9%, and a

secondary infertility incidence of 10.5% (Cong, Li et al. 2016). Increasing male age is a risk factor for infertility. Declining male fertility is related to falling androgen levels, decreased sexual activity, alterations in Sperm motility and morphology, and deterioration in sperm quality and DNA integrity (Sartorius and Nieschlag 2010).

2. Body mass index

According to the researchers, obese women, especially women with abdominal fat hardly become pregnant and have low chance of infertility treatment. In women with a body mass index (BMI) >25, compared with BMI <25, the pregnancy rate is lower (respectively, 10.5% vs. 253%). The correlation between obesity and polycystic ovary syndrome (PCOS) contributes to the infertility in obese women. PCOS is an androgen excess mode with insulin resistance which makes pregnancy difficult. The role of obesity in pregnancy focuses on the physiology of fat body and metabolic disorders. Polycystic ovary is one of the common reasons of ovulation disorder in women of childbearing age. Some studies have shown that usually 30%-70% of women with PCOS are obese. Overweight and obesity in women with PCOS exacerbates the severity of androgen and disorders metabolic profile (Deyhoul, Mohamaddoost et al. 2017). Obesity may disrupt normal folliculogenesis through increased production of IL-10 in visceral fats. This relationship may help clarify the reported association between obesity and ovulatory dysfunction, which has been found in patients with polycystic ovary syndrome (Yang et al. 2021)

3. Duration of infertility

The largest study identified that 85% of women would conceive within 12 months. Based on this study's findings, fecund ability is 25% in the first three months of unprotected intercourse and then decreased to 15% for the remaining nine months. The American Society of Reproductive Medicine

(ASRM) recommends initiating an evaluation for infertility after failing to achieve pregnancy within 12 months of unprotected intercourse or therapeutic donor insemination in women younger than 35 years or within 6 months in women older than 35 (Breitkopf, Hill et al. 2019).

4. Alcohol and smoking

These are modifiable risk factors which are affecting human reproductive function. Smoking is linked to premature menopause in addition to decreased levels of ovarian reserve markers, mediated by an diminishing of antral follicle progress and growth, resultant in cytotoxicity and making of poor quality oocytes. In men alterations in morphology and decreased concentration, motility and viability of Sperm have been observed among smokers (De Angelis *et al*., 2020). Alcohol consumption is associated with higher levels of estrogens and lower levels of progesterone, as well as decreased luteinizing hormone (LH), hCG receptor expression, granulosa cell expression, reduced oviductal smooth muscle cell contractility, irregular menstrual cycles, and ovulatory dysfunction (Akison, Moritz and Reid, 2019). In men shown a significant decline in testosterone levels, seminal fluid volume and sperm concentration in chronic alcoholics (Deyhoul, Mohamaddoost et al. 2017).

5. Infection

Many microbial causes infertility of male and female example Gonorrhea, chlamydia, trichomonas, brucellosis, urea plasma, mycoplasma, coliforms bacteria, adenovirus, enterovirus and mumps (Pai, Venkatesh et al. 2020).

Urogenital infections seem to be one of the major causes of infertility. More than 30 bacterial, viral or parasitic agents are responsible for these infections. Chlamydia, herpes simplex virus (HSV), and human

papillomavirus (HPV) are the most prevalent sexually transmitted infections (STIs). Since urogenital infections are often either asymptomatic or mild symptomatic, they may not be diagnosed by patients and clinicians. Long-term infection can lead to severe reproductive health complications in women, such as stillbirth, preterm delivery, increased risk of HIV acquisition, infertility, cancer, and so on. Untreated Chlamydia trachomatis (C. trachomatis) infection can lead to pelvic inflammation which causes scars inside the reproductive organs and consequently may affect fertility (Pebdeni, Saffari et al. 2023).

6. Chronic disease

In male, the association between male infertility and the risk of the two out of three major nonmalignant chronic conditions according to WHO (diabetes and cardiovascular diseases) and all mortality cause (Glazer, Bonde et al. 2017). In female, many chronic illness associated with female infertility such as, Polycystic Ovaries syndrome Endometrioses and thyroid disorders (Mallikarjuna, Rajeshwari et al. 2015).

Chronic inflammation is a serious medical condition that damages tissues and cells around the body. Recently, chronic inflammation has been identified as a leading cause of death, amounting to about 50% of the global population in cases of neurodegenerative diseases, autoimmune diseases, cardiovascular diseases and cancer (Abay et al. 2018). Chronic inflammation is generally characterized by the colonization of infected or injured sites with monocytes, macrophages and lymphocytes, along with the proliferation of connective tissues and blood vessels. These immune cells produce enzymes and cytokines that can cause long-term damage to cells, tissues and organs. The main systemic features of chronic inflammation include susceptibility to infections, gastrointestinal disorders (e.g. acid reflux, constipation and diarrhea), weight loss or gain, insomnia, prolonged

tiredness, arthralgia, body pain, myalgia, anxiety, depression and mood disorders (Pahwa et al. 2024). The duration and resolution of chronic inflammation are heavily influenced by external factors or internal processes. Among others, external factors include lifestyle (diet and smoking), environmental pollutants and genetics. Chronic inflammation can also be acquired from several autoimmune diseases such as systemic lupus erythematosus (SLE) and type I diabetes. While acute inflammation is a critical and typically temporary response that promotes healing, the persistence of inflammation over time can lead to the development of chronic inflammation, a condition with far-reaching and severe consequences for the body.

1.2.5. Etiology of infertility:

Infertility may occur due to:

-Male factors.

-Female factors.

-A combination of male and female factors.

-Unexplained. For both women and men

However, environmental and lifestyle factors such as age ,smoking, excessive alcohol intake, obesity and exposure to environmental pollutants have been associated with lower fertility rates (Farazi, Hazari et al.). The most common causes of infertility are ovarian dysfunction, tubal disease, and male factor infertility. The remaining 15% of infertile couples experience "unexplained infertility"(Carson and Kallen 2021).

1.2.5.1. Male factor:

Male infertility is defined as the inability of a male to make a fertile female pregnant, also for a minimum of at least one year of unprotected intercourse. The male is solely responsible for about 20% and is a contributing factor in another 30% to 40% of all infertility cases (Cedeno, Light et al. 2023). In several publications, defects in spermatozoa due to inadequate numbers (azoospermia/oligospermia), poor motility, and abnormal structure/morphology have been reported as leading causes of male infertility worldwide. Several causes and risk factors have been speculated and/or proven for male infertility and published in the literature. Some of these include smoking, alcohol intake, drugs, obesity, past or present testicular infections, exposure to environmental toxins, exposure of the testicles to excessive heat, hormonal disorders, testicular trauma and ejaculatory/erectile disorders among several others (Okonofua, Ntoimo et al. 2022).

1.2.5.2. Female factor

According to the Center for Diseases Control (CDC), 1.5 million women in the US (6%) are infertile, and 25% of infertile couples have more than one factor that contributes to their infertility (Salas-Huetos, Rosique-Esteban et al. 2018). The cause of Female infertility is commonly divided into 4 main categories:

I. Hormonal Disorder:

Hormones and inflammatory mechanisms are implicated in the major events of female reproductive function, including ovulation, menstruation, embryo implantation and pregnancy. Increasing evidence shows that hormonal aberrations and a hyperinflammatory state may lead to derangements of the immune-endocrine cross talk among endometrium,

myometrium and cervix, and between the decidua and trophoblast, predisposing to pregnancy complications. Reproductive disorders (endometriosis, adenomyosis, polycystic ovary syndrome and uterine fibroids) and unexplained infertility share inflammatory pathways, hormonal aberrations, decidual senescence and vascular abnormalities that may impair pregnancy success through common mechanisms. Either in combination or alone, these disorders result in an increased risk of preterm birth, fetal growth restriction, placental pathologies and hypertensive disorders. Systemic hormonal aberrations, and inflammatory and metabolic factors acting on endometrium, myometrium, cervix and placenta are all associated with an aberrant milieu during implantation and pregnancy, thus contributing to the genesis of obstetric complications. Some of these features have been also described in placentas from ART. (Vannuccini, Luisi et al. 2018).

II. Ovarian Disorders

Ovulatory disorders represent a major cause of infertility. The World Health Organization classification offers a useful frame for diagnosis and treatment. Polycystic ovary syndrome (PCOS) is the most common cause of oligoovulation and anovulation (Balaban and Urman 2006). PCOS is a complex endocrine and metabolic disorder, typically characterized by anovulation, infertility, obesity, insulin resistance, and polycystic ovaries. Lifestyle or diet, environmental pollutants, genetics, gut dysbiosis, neuroendocrine alterations, and obesity are among the risk factors that predispose females to PCOS. These factors might contribute to upsurging metabolic syndrome by causing hyperinsulinemia, oxidative stress, hyperandrogenism, impaired folliculogenesis, and irregular menstrual cycles. Dysbiosis of gut microbiota may play a pathogenic role in the development of PCOS. The restoration of gut microbiota by probiotics,

prebiotics, or a fecal microbiota transplant (FMT) might serve as an innovative, efficient, and noninvasive way to prevent and mitigate PCOS. This review deliberates on the variety of risk factors potentially involved in the etiology, prevalence, and modulation of PCOS, in addition to plausible therapeutic interventions, including miRNA therapy and the eubiosis of gut microbiota, that may help treat and manage PCOS (Singh, Verma et al, 2023)

III. Fallopian Tube Damage or Blockage

The fallopian tube plays an important role in the mechanical transport and physiological sustenance of the gametes and early conceptus. Complex and coordinated neuromuscular activity, cilial action and endocrine secretions are required for successful tubal function. Compromised tubal damage can occur after external or internal injury, inhibiting the normal transport of gametes. The overall prognosis for fertility depends principally on the insult and the severity of the tissue damage; hence, assessment of tubal damage plays a major role in predicting occurrence of pregnancy and the likelihood of developing ectopic pregnancy (Patil 2009).

IV. Uterine Factors:

Uterine factor infertility (UFI) is defined as a complete lack of a uterus (Absolute Uterine Factor Infertility or AUFI) or as a nonfunctional uterus (Non-Absolute Uterine Factor Infertility or NAUFI). The exact prevalence of UFI is currently unknown. Early studies (1970s), which have been repeatedly conducted over the years, suggest that it affects 3– 5% of the world's female population and that AUFI affects up to 1 in 500 women of childbearing age.

There are many causes of UFI, congenital and acquired. uterine agenesis, hysterectomies, uterine malformations, polyps, myomas, adenomyosis, synechiae, uterine irradiation (Sallee, Margueritte et al. 2022).

1.2.5.3. Combination factors:

In 30-15% of couples, both the male and female are infertile (Mustafa, Sharifa et al. 2019).

1.2.5.4. Unexplained infertility:

Fertility testing hasn't found a reason that a person or couple is unable to get pregnant (Penzias, Bendikson et al. 2020). The failure of conception not explained by anovulation, poor sperm quality, tubal pathology or any other causes of infertility, the two most useful treatments for unexplained infertility are intra-uterine insemination and in vitro fertilization (Mol, Tjon-Kon-Fat et al. 2018). In 15–30% of patients, infertility remains unexplained (La Marca and Mastellari 2020).

1.2.6. Treatment modalities of infertility:

Infertility treatment depends on the cause, duration, both partners age, and personal preferences. The couple should be explained that some of the causes of infertility cannot be corrected. Financial, physical, and time commitment is required for infertility treatment (Anwar and Anwar 2016).

Infertility is typically treated with fertility drugs, medical procedures, surgery, or a combination of these (Dayan, Joseph et al. 2019).

Assisted Reproductive Technology are a group of medical procedures for treating the infertile human, including:

1.2.6.1. Intra-uterine Insemination:

IUI is a treatment often used for couples with unexplained subfertility. In an IUI cycle, the male partner's sperm is prepared and placed directly in

the womb around the time of ovulation. IUI cycles can be used in combination with fertility drugs to increase the number of available eggs. However, these drugs can have side effects, and also increase the risk of multiple pregnancies. Expectant management and timed intercourse have also been shown to increase pregnancy rates, resulting in live births (Ayeleke, Asseler et al. 2020).

1.2.6.2. In vitro fertilization (IVF):

In IVF, multiple mature eggs from a woman are retrieved, and fertilized with a man's sperm outside the uterus and inside a laboratory. Then, the fertilized embryos are implanted in the uterus after three to five days of fertilization (Bain 2019).

1.2.6.3. Zygote Intra-fallopian Transfer (ZIFT) and Gamete Intrafallopian Transfer (GIFT):

In ZIFT, the fertilized egg is directly transferred into the fallopian tube; whereas, in GIFT a mixture of sperms and eggs is placed in the fallopian tube and fertilization occurs there (De Jongh 2019).

1.2.6.4. Intracytoplasmic Sperm Injection (ICSI): a technique where a single sporozoan was injected mechanically into an oocyte in vitro to achieve fertilization, was introduced (Zheng, Zeng et al. 2019).

1.2.6.5. Assisted hatching: Through this technique, implantation of the embryo into the uterus is assisted by breaking the outer covering of the embryo. This helps the embryo to smoothly implant (Hammadeh, Fischer-Hammadeh et al. 2011).

1.2.7. Intracytoplasmic sperm injection, or ICSI:

The Intracytoplasmic sperm injection (ICSI) is a micromanipulation technique that allows the injection of one spermatozoon into the ooplasm of a metaphase II oocyte. In humans, ICSI has been utilized for more than a

quarter of a century and has become one of the most widely used assisted reproductive technologies for reproduction in humans. The widespread use of ICSI is the result of the capacity for this procedure to be utilized in addressing many reproductive problems, such as lack of sperm motility and globozoospermia, which are mainly related to male infertility. Furthermore, ICSI has been utilized to overcome some problems when there is female infertility, such as less-than-optimal quality oocytes and/or when it is only possible to collect a small number of oocytes (Briski and Salamone 2022).

1.2.7.1. Indication of ICSI:

A) Male Factors Infertility: Include

1- Severe oligospermia refers to a low spermatozoa count.

2. Severe asthenozoospermia causes impaired motility.

3. Abnormal morphology (severe teratozoospermia).

4- Combination of all (Severe Oligoasthenoterozoospermia).

5- In cases of azoospermia, testicular sperm extraction (TESE or micro-TESE) can be used to obtain spermatozoa.(Lacey, Henderson et al. 2021).

6. When the neck of the bladder does not shut after expulsion, semen refluxes into the bladder, causing retrograde ejaculation.

7. Examine electroejaculation in males who do not ejaculate or who have anomalies due to neurologic impairment.

8. Immunological infertility may result from anti-sperm antibodies in either partner (Haddad, Stewart et al. 2021).

B) Non-male factor indication:

1- Fertilization of poor-quality or dysmorphic oocytes.

2- Poor responders to maximize fertilization rate.

3- Cryopreservation oocytes or sperm to postpone conception.

4- In conjunction with preimplantation genetic testing (PGT) to evaluate the genetic status of embryos in addition to increase the likelihood of implantation.

5- Advanced maternal age patients and Unexplained infertility (Pereira, Palermo et al. 2018).

1.2.8. Follicular Fluid (FF):

Follicular fluid (FF) surrounds the granulosa cell-oocyte complex and is one of the mediating factors in the communication between the cells within the follicle. Literature reveals that human FF and its components are key factors to the success of natural fertilization. Among other substances, FF consists of multiple cytokines and immune cells, including interleukins, macrophages, NK cells and lymphocytes. Together, these cells and cytokines might influence the oocyte-granulosa-cell complex. Altered balances of immune content might be involved in changes on folliculogenesis, oocyte maturation, oocyte quality and ovulation (Prins, Marissen et al. 2020).

These altered balances are possibly involved in infertility associated with immune-mediated diseases such as PCOS and POR. The human follicular fluid which is the microenvironment of the oocyte during its development and maturation, is a semi-viscous, hypo coagulable fluid comprising a wide variety of biologically active molecules. It is a product of transfer of plasma constituents across the blood-follicular barrier and the secretory activity of granulosa and theca cells. It is important in ovarian physiology, including steroidogenesis, growth of the follicle and ovulation, maturation of the oocyte and its transport to the oviduct (Wu, Wu et al. 2015, Usman, Shuaibu et al. 2021). It can be obtained by trans-vaginal oocyte retrieval for in-vitro fertilization (IVF). It is extracellular fluid that forms during development of the antral follicle under the influence of follicle

stimulating hormone (FSH). FF bathes the developing oocyte. It is constituents can impact oocyte development and maturation, necessary events for fertilization and eventually live birth. A majority of an embryo's cytoplasm and a substantial portion of it is DNA is derived from the oocyte. As such, FF measurements have the potential to offer greater insight into biologically effective doses affecting fecundity than either blood or urine (Butts 2020).

However, FF obtain only by invasive techniques for in vitro Analysis. Therefore, an oocyte pick-up (OPU) is required, which is an Invasive procedure, there is no possibility of leaving the developing egg in Vivo and un-disturb it, FF is essential in ovarian physiology, and steroidogenesis, development of the follicles, maturation of the oocytes, ovulation, As well as their transport to the oviduct (Güngör and Güngör 2021).

1.2.9. Immunological markers of infertility

1.2.9.1. The cytokines

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 (L Field et a, 2014).

Cytokines were originally identified as products of immune system cells and are important immune response mediators. Cytokines can stimulate or inhibit cell growth, regulate cell differentiation, induce cell chemotaxis, and modulate the expression of other cytokines (Günther, Alkatout et al. 2016). Cytokines have been found to regulate the reproductive process by influencing the immune environment within the follicle itself, as well as the uterus (Lu *et al.*, 2018). It consists of smaller water-soluble proteins and glycoproteins. They are classified into lymphokines, interleukins, and

chemokines based on their function, cell origin, and target cells. Cytokines can modulate the function of immune cells within the ovary in the context of reproduction. (Prince *et al*., 2020)

Cytokines can modulate the function of immune cells within the ovary in the context of reproduction. The ovarian interleukins, which are secreted by the granulosa cells and other immune cells within the ovaries and follicles along with hormonal changes, regulate various functions, including folliculogenesis, oogenesis, ovulation, fertilization, embryonic development, implantation, formation, and regression of the corpus luteum (Gavrilovic, Cekovic et al. 2022). One of the success factors of in vitro fertilization (IVF) is the quality of the obtained oocytes. The quality of the oocyte is significantly affected by the environment in which it is located or the so-called microenvironment. Defining certain parameters of the microenvironment, which can be easily and quickly detected, which enable the differentiation of oocytes that are of better or worse quality, could potentially increase the success of IVF. In the follicles, oocytes undergo growth and maturation. The follicular wall consists of granulosa and theca cells, which are separated by the basal membrane. The maturation process is carried out through several stages within follicles. During follicular growth, its interior is filled with follicular fluid that is made by the filtration of the blood plasma constituents and by the secretory activity of granulosa and theca cells (Prins, Marissen et al. 2020).

1.2.9.2. Role of some cytokines in the infertility:

1.2.9.2.1. Interleukin-6 (IL-6)

(IL_6) is a pleiotropic cytokine with multiple effects that can vary depending on the physiological environment. IL-6 promotes T cell population expansion, B cell differentiation, and proliferation of many cells it can also regulate the acute phase response and various homeostasis

functions, including glucose metabolism, lipid metabolism, and insulin resistance. IL-6 also affects the endocrine and nervous systems. It is also involved in the occurrence and development of various cancers. IL-6 secrete by macrophage, dendritic cell, neutrophils and B cell. IL-6 also plays an important role in follicle development. Regarding folliculogenesis, cytokines regulate cell proliferation or differentiation, follicle survival or atresia, and oocyte maturation (Gavrilovic, Cekovic et al. 2022). In the poly-cystic ovary syndrome, IL-6 levels increase in the serum as well as in the follicular fluid due to an increased secretion by the granulosa cells, and some studies have shown that increased IL- 6 levels may be associated with hyperandrogenism and insulin resistance (Zhuang, Pan et al. 2019).

Some studies have shown that High levels of IL-6 are associated with an increased rate of clinical pregnancies and implantation of embryos (Wu, Bersinger et al. 2017).

However, other studies have reported contradictory results. Higher levels of IL-6 correlate with poor embryo quality, and patients are less likely to become pregnant (Altun, Jindal et al. 2011). It has been concluded that IL-6 inhibits oocyte development (Banerjee, Sharma et al. 2012). Furthermore, some research indicates that IL-6 has no effect on the clinical rate of pregnancy or the oocyte quality (Alhilali, Parham et al. 2020). Other studies have confirmed this Significant difference in serum IL-6 levels between unexplained infertile and fertile women suggests that this cytokine may be involved in pathophysiology of unexplained infertility (Demir, Guven et al. 2009).

As an inflammatory factor, IL-6 is synthesized and secreted by granulosa and immune cells in the ovary, and plays a key role in the physiological function and pathological progression of the ovary. In terms of physiological functions, IL-6 is involved in the development and maturation of ovarian

follicles, ovulation, hormone secretion and other key physiological processes, as one of the cytokines that play an essential regulatory role in the ovary. In the pathological process, the overexpression of IL-6 interferes with the normal physiological process of the ovary, as a marker of inflammation in the ovary. IL-6 can also reflect the level of endocrine disorder and oxidative stress in patients. Studies on IL-6 related genes have further elucidated the close correlation between IL-6 and ovarian pathology. Targeting IL-6 to treat some abnormalities in the ovaries is promising (Tan and wang 2024).

1.2.8.2.2: Interleukin-10 (IL-10)

Is a potent anti-inflammatory cytokine that plays a crucial, and often essential, role in preventing inflammatory and autoimmune pathologies, secreted by myeloid cells like macrophages, monocytes, dendritic cells, neutrophils, eosinophils and mast cells (Iyer and Cheng 2012). Some studies showed significant decrease in their levels in the infertile women when compared with the fertile women (Khalid, Ahmed et al. 2022). The degree of preconception IL-10 evation may correlate with an increased risk of IVF failure. Elevated IL-10 ratios can be corrected with therapy. It may be possible to improve IVF success rates by modulating high cytokine levels (Winger and Reed 2011).

Other study revealed notable differences in serum IL-10 levels between PCOS and non-PCOS groups, suggesting a unique immunological profile in PCOS patients undergoing fertility treatments. This cytokine did not significantly correlate with the total number of retrieved oocytes, oocyte maturity, fertilization rate, or pregnancy rate (Albayati and Abdulhameed 2024).

1.2.8.2.3 Endothelial gland-derived vascular endothelial growth factor (EG-VEGF)

Is secreted by steroidogenic cell in endocrine glands. Also termed as prokineticin (PK1), is the first protein identified as a selective angiogenic mitogen acting on the endothelial cells of steroidogenic endocrine glands serum concentrations of EG-VEGF were associated with the pregnancy outcome. So EG-VEGF may be applicable as an indicator for IVF pregnancy outcome, but more experimental supports such as EG-VEGF expression in endometrium during WOI phase are needed. Moreover, as serum concentrations of EG-VEGF may vary during the luteal phase, further study with sampling at multiple timepoints will assure its role to predict the clinical pregnancy outcome (Gao, Zhao et al. 2012). EG-VEGF acts through its two G-protein-coupled receptors PRK1 and PRK2 (pro-kineticin receptor 1 and 2). Stimulation of these receptors activates calcium mobilization, leading to smooth muscle contractions and angiogenesis (Lin, LeCouter et al. 2002).

Chapter Two

Materials and Methods

2. Study design, Materials and Methods

2.1. Study design

The cross-sectional study was done in Al-Kafeel Super- specialty hospital in Karbala province from February 2024 to June 2024.

2.1.1. Subjects

The study included 37 subjects divided into four groups depended on the four common causes of infertility: Unexplained infertility, male factor and female factor.

2.1.2. Exclusion criteria:

1. Female factor due to endometriosis.
2. Cycles end with ovarian hyper stimulation syndrome.
3. Cancelled cycles.
4. Azoospermia patients or sperm source from testicular biopsy.
5. Very semen sperms abnormity.

2.1.3. Inclusion criteria:

1-Infertile female their age between (18-40years).

2-All cycles that end with ova pick up and sperm injections and fresh embryo transfer.

2.1.4. Ethical issue

Ethical approval was obtained from Karbala Medical College Ethical Committee. Also, verbal approval was taken from the patients before taking on the sample. During sample collection, health measures and safety was taken.

2.2. Materials

2.2.1. Equipment and Instruments

The main instruments and Equipment used in this study are listed in the table (2-1).

Table (2-1): Instruments and Equipment

Equipment and instruments	Company	Source
Air coda	GenX	USA
Air incubator	Heraeus, Kelvitrout®	Germany
Automatic pipette	Thermo-scientific	USA
Center well dish	Thermo scientific	USA
Centrifuge	Universal 16 A	Germany
CO2 incubator	Genx International	USA
CO2 incubator analyser	GEOTEC	India
Codan syringe 1 ml	Codan	Denmark
Conical tube	Falcon	USA
Deep freeze refrigerator	New Brunswick	UK
Disposable syringe (5ml)	Medi	China
Disposable Tips for automatic pipette Finntip	Thermo scientific	USA
Double lumen	GYNETICS	Belgium
ELISA automated washer	Paramedical	Italy
ELISA reader and printer	Paramedical	Italy
Embryo transfer catheter	GYNETICS	Belgium
Eppendrof tubes (0.2& 1.5 ml)	Sterellin Ltd	UK
EZ tip (155-135)	RI	UK

Face mask	PRO. Care	China
Falcon® Tissue Culture Flasks	Falcon	USA
Four 4- well dish	Nunc	Denmark
Gel Tube	AFCOVAC	Jordan
Holding pipette for ICSI	Cook	Australia
Injecting needle for ICSI	Cook	Australia
Inverted microscope	Olympus Optical Co Ltd.	Japan
Laminar air flow hood	Gelman instrument	Germany
Latex gloves	Comfit	Malaysia
Light Microscope Olympus	Optical Co Ltd.	Japan
Micromanipulator	Narshiege	Japan
Micropipette (different size)	Eppendorf	Germany
Micropipette tips (different sizes)	Human	Germany
Microscopical slides and cover slides	Marienfeld	Germany
MINI VIDAS®	BioMérieux	France
Petri Dish 90 cm	Nunc	Denmark
Petri Dish 60 cm	Nunc	Denmark
Plane test tube	ALS	China
Plastic pasture pipette	India MART	India
Refrigerator	Concord	Lebanon
Round bottom tube 14 mL	Nunc	China

Thermometer	Thermo scientific	USA
Ultrasound device	MediSON	Korea
Water pump (Aspirator 3)	LABOTECT	Germany

2.2.2. Chemical and biological materials

The main chemicals used in this study are in table (2-2).

Table (2-2): Chemical and biological materials with their Manufacturing company and country of origin

Chemicals and biological materials	Manufacturing Company	Country
Aspiration media	Fertipr	Belgiu
Gain Early stage media	Fertipro	Belgiu
HEPES media	Fertipro	Belgium
Hyaluronidase	Fertipro	Belgium
Mineral oil	Fertipro	Belgium
Oosafe® Disinfectant of CO2 incubator	SparMCD	Denmark
Oosafe® hand Disinfectant	SparMCD	Denmark
Polyvinylpyrrolidone 10% (PVP)	Fertipro	Belgium

2.2.3. ELISA Kit used in the study

ELISA Kit used in this study are listed in the table (2-3).

Table (2-3): ELISA Kits used in the study

ELISA Kit & Catalog No.	Manufacturing Company	Country
1.HumanIL-6 (Interleukin 6) ELISA Kit Catalog No: E0090H4 2.HumanIL-10 (Interleukin 10) ELISA Kit Catalog No: Eolo2Hu 3.Human Endocrine gland vascular endothelial growth factor (EG-VEGF) ELISA Kit Catalog No : E018H4	BT LAB	China

2.2.3.1. ELISA Kit Content of Human IL-6, IL-10 and EG-VEGF

The kit of ELISA contents used in this study is listed in table (2-4).

Table (2-4): Kit components and Storage of IL-6, IL-10 and EG-VEGF

Components	Quantity (96T)	Quantity (48T)
Standard Solution (2400ng/L)	**0.5ml x1**	**0.5ml x1**
Pre-coated ELISA Plate	**12 * 8 well strips x1**	**12 * 4 well strips x1**
Standard Diluent	**3ml x1**	**3ml x1**
Streptavidin-HRP	**6ml x1**	**3ml x1**
Stop Solution	**6ml x1**	**3ml x1**
Substrate Solution A	**6ml x1**	**3ml x1**
Substrate Solution B	**6ml x1**	**3ml x1**

Wash Buffer Concentrate (25x)	**20ml x1**	**20ml x1**
Biotinylated Human EG-VEGF Antibody	**1ml x1**	**1ml x1**
User Instruction	**1**	**1**
Plate Sealer	**2 pics**	**2 pics**
Zipper bag	**1 pic**	**1 pic**

2.3 Methods

2.3.1 Sample Collection

At day of Oocytes retrieval, Serum and follicular fluid samples were collected.

2.3.1.1 Follicle Fluid (FF):

Follicular fluid was obtained from ovarian follicles at the time of oocyte pick up, with ultrasound guided aspiration needle and emptied into planed tube. Transparent Follicular fluid collected after removal of oocytes from collection step in ICSI lab into plane tube centrifuged immediately at 15 min for. 3000 rpm and taken to get out of the debris and the supernatant stored at -20°C for an ELISA test to determine the concentration of IL-6, IL-10 and EG-VEGF.

2.3.1.2 Serum Sample:

3 ml of Blood samples were drawn from the veins of 37 subjects at the day of Oocytes Retrieval by using a disposable syringe and a sterilization technique. Blood was collected in gel tube blood was allowed to clot for 15 min after that serum separated by centrifugation at 3000 rpm for 5 minutes. Two ml of serum was collected in an Eppendorf tube and then stored at -20 °C for an ELISA test to determine the concentration of IL-6, IL-10 and EG-VEGF.

2.3.2. Estimation of IL-6, IL-10 and EG-VEGF concentration in serum and follicular fluid

2.3.2.1. Test principle of IL-6, IL-10 and EG-VEGF

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human IL-10 antibody. IL-10 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human IL-10 Antibody is added and binds to IL-10 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated IL10 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human IL-10. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.3.2.2. Reagent Preparation

1-All reagents she was in room temperature before use.

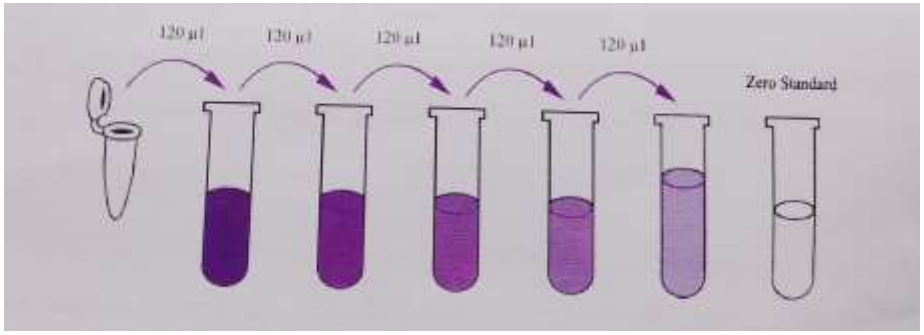
2-Standard Reconstituted the 120µl of the standard (1600pg/ml) with 120µl of standard diluent to generate an 800pg/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (800pg/ml) 1:2 with standard diluent to produce 400pg/ml, 200pg/ml, 100pg/ml and 50pg/ml solutions. Standard diluent serves as the zero standard (0 pg/ml). Any remaining solution should be frozen at -20°C and used within one month.

Dilution of standard solutions suggested are as follows:

Table (2-5) : Dilution of standard solutions

1200ng/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
600ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
300ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent

150ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
75ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
1600pg/ml	800pg/ml	400pg/ml	200pg/ml	100pg/ml	50pg/ml

Figure(2-1): Dilution of standard solutions

3-Wash Buffer Diluted 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

2.3.2.3. Assay procedure

1. Prepared all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2. Determined the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.

3. Added 50µl standard to standard well. Note: Don't add biotinylated antibody to standard well because the standard solution contains biotinylated antibody.

4. Added 40µl sample to sample wells and then add 10µl anti-IL-10 antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer, Incubate 60 minutes at 37°C.

5. Removed the sealer and wash the plate 5 times with wash buffer. Soak wells with 300ul was buffer for 30 seconds to I minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material

6. Added 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7. Added 50µl Stop Solution to each well, the blue color will change into yellow immediately.

8. Determined the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.3.2.4. Results Calculation

Constructed a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and drawed a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

2.3.3. Female Preparation for ICSI

2.3.3.1. Controlled Ovarian Hyper-stimulation (COH)

On day two of female cycle checked the female patients, by vaginal ultrasound to evaluate the antral follicles count (follicles with diameter of 210mm), to rule out any ovarian cyst, and to measure the endometrial thickness. In the same day, the Gynecologists sent the patient for hormonal analysis including FSH, LH, Prolactin, E2 and AMH with mini VIDAS immunoassay technique, then

according to the finding on examination, U/S, hormonal analysis and patient's information, they underwent COH (Malathi, Balakrishnan et al. 2021).

*****Controlled ovarian hyper stimulation (COH) comprises 3 basic elements:

- Exogenous gonadotrophins to stimulate multiple follicular maturation. - Co-treatment by either gonadotropin-releasing hormone (GnRH) agonist or antagonists to suppress pituitary action and inhibit premature LH surge. - Triggering of final oocyte maturation (36 to 38) hours prior to oocyte Retrieval (Gallos, Eapen et al. 2017).

2.3.3.2. Measurement of E2 for Follow up

The serial measurement of E2 level (same process mentioned before) was used to evaluate the patient's response to the treatment together with endometrial thickness and follicles number. This help in the estimation of the day of hCG injection.

Antagonist protocol:

All patients in this study underwent for GnRH antagonists (cetrotide0.25 mg, Merck Serono) involve a shorter duration of use, absence of vasomotor symptoms, minimizing of ovarian hyper-stimulation syndrome, and a significant lower dose of gonadotropin per cycle, make

GnRH antagonists not only well-tolerated by patients but also clinicians', and are started a few days after initiation of stimulation, until day of hCG injection (Eftekhar and Tabibnejad 2021).

Ovulation Triggers:

By human chorionic gonadotropin (hCG) (Lupi-HCG 10,000 IU) trigger shot intramuscularly when at least 2 dominant follicles have reached 18–20 mm, which is used to mimic the natural endogenous LH surge to initiate the process of final oocyte maturation and better timing before ovulation around 36 hours (Salama, Sakr et al. 2021).

2.3.3.3. Oocyte Pick Up

Which done by gynecologist occur within 36-38 hours after ovulation induction, by trans-vaginal ultrasound-guided trans-vaginal puncture that passed through vaginal wall to the ovary for oocyte retrieval, this was done under general anesthesia. One end of the needle was attached to suction pump which creates a negative pressure not exceeded (140 – 150) mmhg to avoid oocyte rupture for aspirating follicles. "Flushing tube with 5ml syringe holding aspiration medium (HEPES, heparin containing) was utilized for this purpose (De Roo and Tilleman 2021). Follicular fluid collection by Prewarmed tubes and these tubes then transferred to IVF lab for cumulus oocyte complex (COC) searching and collection by Embryologist, by using a Petri dish and 1 ml syringe with adapter For searching of COC under dissecting Stereomicroscope then the COC transferred to a four-well dish Containing culture medium, which was prepared overnight and incubated at 37ċ with 5% CO2 till time of oocyte denudation (ART, D'Angelo et al. 2019).

2.3.4. Assisted Reproduction Technique

2.3.4.1. Dishes Preparation

1. Preparation of collection Dish for ICSI

Four wells dish filled by 600 µl of Hydroxy ethyl piperazine ethan sulfonic acid (HEPES) media each well and covered by mineral oil ,prepared and incubated overnight in incubator conditions of 5% Co2 and 37ºC (Johansson and Embryos 2014).

2. Preparation of Denudation Dish

A drop of about (100-200) µl of hyaluronidase solution with multiple drops (100-200) µl of flashing media were placed on a petri dish and placed in the hood on heated area to keep it warm. Stripper tips must be prepared with inner diameters of 290, 170,155 and 135 µm respectively (Naji, Moska et al. 2018).

3.Preparation of Injection Dish

Number of drops according to oocytes number, each one is 5 µL of

HEPES buffered medium, arranged in a shallow falcon dish, add 5 µL of Polyvinylpyrrolidone 10% (PVP) to the central droplet and covered with oil the sperm will be placed in it to decrease sperm movement due to PVP viscous nature, so facilitating sperm manipulation. The dish was placed on heated area in the hood (Johansson and Embryos 2014).

4. Culture Dish

A special IVF media with mineral oil were incubated in culture dishes overnight in incubator conditions of 5% Co2 and 37ºC. The culture system for embryo includes droplets under oil: 5 drops of culture media of 50-µl volume were arranged with one central drops surrounded by four drops covered by 5 ml of mineral oil then placed in the incubator (Johansson and Embryos 2014).

2.3.4.2. Collection of Oocytes on the Day of Oocytes Retrieval

In ICSI lab, the tubes received containing the follicular fluid to look for the oocytes. For the collection of COC, the embryologist had already prepared an adaptor, a 1 ml syringe(codon), a petri dish (90cm) and 4- well dish (A dish prepared in advance the day before). The scanning for COC had been achieved under a stereoscopic dissecting microscope. The petri dish was examined under a low power lens (6x-12x) while the confirmation of the oocyte presence had been done under a higher magnification (25x-50x) by the fining of COC under dissecting stereomicroscope. Oocytes isolated in 4-well dish and kept incubator.

2.3.4.3 Oocyte Denudation

Oocytes are enclosed by the cumulus cells forming COC these COC may block the injector needle and so interfere with the gentle oocyte microinjection. Furthermore, because only mature oocytes that have reached the MII stage are appropriate for ICSI, ideal optical circumstances to allow assessment of the meiotic status of the oocytes are necessary, and these become restricted in the existence of the cumulus cells (Naji, Moska et al. 2018).

Denudation done by two steps:

A. Enzymatic Denudation: Because of one of the main components of COC is hyaluronic acid so hyaluronidases enzyme is usually used for removal of glycol-protein granules of the hyaluronic matrix so, low concentrations of the enzyme for instance 80 IU/ml is used (no more than 1 minute).

B. Mechanical Denudation: Further denudation was done mechanically by gentle pipetting through capillaries of successively narrower inner diameters of 290, 170, 155 and 135 µm respectively in enzyme free HEPES -buffered media, then oocytes washed off and all cumulus cells had been detached.

Lastly, the denuded oocytes were transferred to the 4 well dish droplets and their meiotic status and morphology were estimated (Carvalho, Leal et al. 2020).

2.3.4.4 Evaluation of Oocyte Denuded

Under an inverted microscope, the morphologic features of the oocytes were classified according to the presence of intra- and extra-cytoplasmic abnormalities, as well as whether they were germinal vesicle GV (immature with oocyte nucleus), MI (immature no pole body), or MII (mature with polar body).(Faramarzi, Khalili et al. 2017).

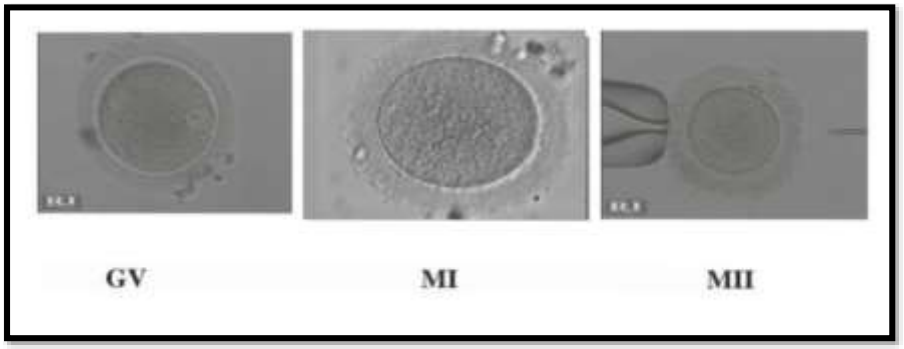


Figure (2-2) shows the maturity of GV, MI, and MII oocytes in an ICSI plate at a magnification of 40X using an inverted microscope.

2.3.4.5 Sperm Preparation:

The most widely used method for the sperm's preparation for ICSI was the Centrifugation swim up technique. It is summarized in the following steps:

- After abstinence time between 2-7 days, the semen was collected by masturbation in special room near the IVF lab and ejaculated into wide mouth, clean container labeled with the name, age of patient and the time of collection. The patients were instructed to collect the whole sample.
- The sample was left at room temperature for liquefaction completely for about 30-60 minutes. The macroscopic and microscopic examination were done.
- After completing the liquefaction, the sample then was transferred to conical test tube, to be diluted with HEPES-buffered media, and centrifuged.
- After centrifugation, the supernatant was discarded and the pellet overlaid with HEPES media.
- The conical tube was incubated at 30-45 minutes.

After the incubation time, the sample need aspiration the upper half (McVay, J. et al. 2021).

2.3.4.6. Set up of the ICSI Micromanipulator

An inverted microscope equipped with micromau: pulator for microinjector pipette and micro-holder pipette used for ICSI procedure with a special heated stage to maintain work temperature at 37ºC. The two manipulators permit three-dimensional movement. The micro-holder was used for fixing and releasing the oocyte while the micro-injector pipette used for immobilization of sperms and injecting the sperm inside the oocyte.

2.3.4.7. Sperm Immobilization

The sperm as much as possible of normal morphology and motility was selected to be immobilized. The immobilization had been done by mechanical pressure of the sperm against the flour of the ICSI dish. The sperm was place at 90 degrees to the tip of the injector. Then, the injector was lowered to compress the sperm tail (figure 2-3a). Perfectly immobilized sperm should keep the shape of the tail normal. If any damage or kinking happened to the tail, the sperm was discarded and the procedure was repeated to another sperm. (Fleming and Oocyte 2021).

2.3.4.8. Oocyte Injection (Day 0)

The polar body was placed at six o'clock, and the oocyte was held in place by pipette suction force to prevent damage to the meiotic spindle. The immobilized sperm was already near the tip of the injector, which was brought close to the oocyte being held and injected into the ooplasm of the MII egg at three o'clock. The least amount of suction was required to allow the ooplasm to enter the injection pipette.

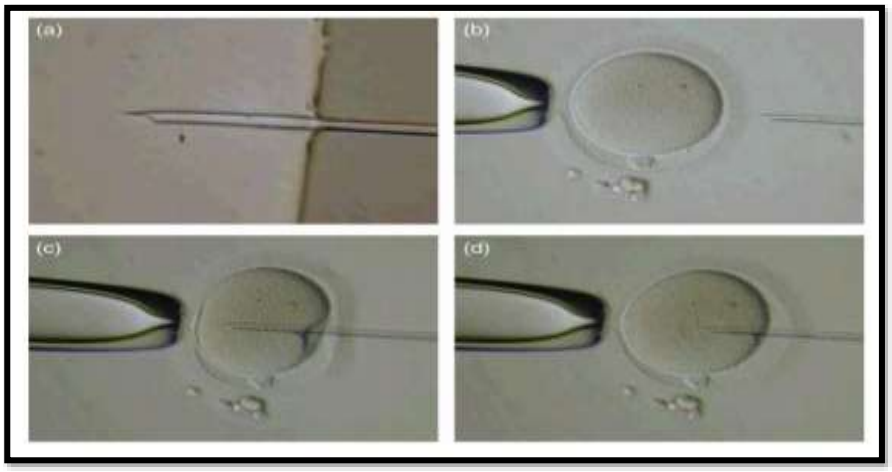


Figure 2-3: Intracytoplasmic sperm injection (b, c, and d) and sperm immobilization (a) of embryos. (at six o'clock, in an ICSI plate with an inverted microscope and a 40X magnification.)

2.3.4.9. Incubation of the Injected Oocyte (Day 0)

After the injection, the oocytes had been cultured and incubated in droplet of culture media covered with mineral or paraffin oil. Oocytes then kept in atmosphere of 5% Co2 and 37ºC (Sciorio, Rinaudo et al. 2023).

2.3.4.10. Assessment of Fertilization (Day 1)

A After 16-18 hour of ICSI, the injected oocytes were checked for intactness and fertilization. The normally fertilized oocyte should have two polar bodies along with two visible pronuclei, which have the nucleoli (Tepla, Topurko et al. 2022) as demonstrated in figure (2-4).

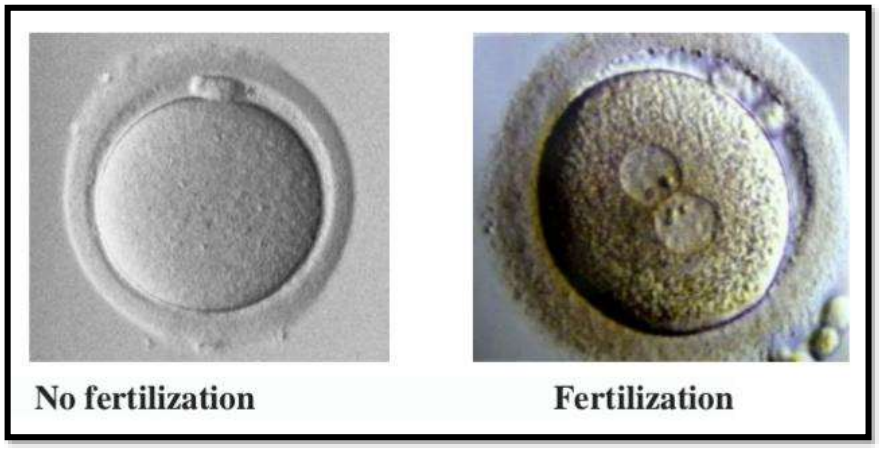


Figure 2-4: Evaluation of oocyte fertilization (in an ICSI plate at 400X magnification using an inverted microscope).

2.3.4.11. Assessment of Embryo Quality (Day2-3)

After assessing the oocytes for fertilization, the morphological evaluation of embryo occurred around 48 hours subsequent to oocyte pick up depending on: cleavage, symmetry of blastomeres, and presence and percentage of fragmentation and state of nucleation. Next to ICSI, more than 90% of 2-PN zygotes will enter cleavage, leading to multicellular embryos. If the embryo was of a good quality, it will reach four cell stages on day 2 and 8 cell stage on day 3

after injection. The resulting embryos are scored according to the number of blastomeres, how equal in size are they and the percentage of fragmentation (Eastick 2022).

2.3.4.12. Embryo Transfer

The human embryo was chosen for transfer depending on its morphology at the zygote, cleavage, and blastocyst stage. Good quality embryos were surely the best for transfer firstly. The embryos with grade A (good quality) and B (fair quality) have been transferred to the uterus while other embryos without upward grading system were left over. 48-72 hours after oocyte pick up, the best embryos 'around three' were transferred. The loading catheter (inner part) was bathed two time in the transfer media then loaded as follows: 5-7 microliter of air, then ~20 microliter of transferring media together with the embryos that is often bracketed with air, and finally 5-7 microliter of media for sealing the catheter. The catheter with the embryos were given to the gynecologist to transfer the embryos gently to the uterus where the outer part was cited in the uterus. This was followed by pulling out the whole catheter and checking it for retained embryos (Swadi, Edan et al. 2023).

2.3.5. Statistical analysis

Statistical analyses were performed using SPSS statistical package for Social Sciences (version 20.0 for windows, SPSS, Chicago, IL, USA). Quantitative data are represented as mean, SD for the normal distribution data. ANOVA test (analysis of variance) and LSD (least significant difference) was used to test the difference between the three studied groups in the normal distribution data. Student's t-test was used to study the difference between two groups. Chi-square test was used to test the relation between qualitative data.

Pearson's correlation test was used to test the relation between cytokines and studied clinical characteristics.

P value of <0.5 was considered as significant.

Chapter Three
Results

3. Result:

3.1. Demographic characteristics among studied groups according to the infertility causes:

Demographic characteristics of the subjects according to the cause of infertility are presented in table (3-1).

The ages of patients ranged from 20-41 years. The mean ages of women with unexplained infertility, male factor, female factor and combined factor were 29.6, 29.6, 32.4, 31.6 years, respectively. There was no significant difference between the patient groups (*P* =0.131) regarding age. The mean of body max index (BMI) in patients with unexplained, male, female and combined factor infertility 25.5, 27.5, 32.4 and 31.6, respectively. There was no significant difference between the patient groups (P=0.079) regarding body mass index. About duration of infertility, the mean values of unexplained infertility, male factor, female factor and combined factor were 11.2, 10.2, 11 and 8.3 years respectively with a non-significant difference (P= 0.156).

Concerning the types of infertility, the unexplained infertility count of primary infertility was 3, while the counts of male, female and combined factors were 9, 4 and 6 respectively. Whereas, the unexplained infertility count of secondary infertility was 2, while the counts of male, female and combined factors were 10, 1 and 2 respectively There was no significant differences about infertility types between patients' groups (*P* =0.121).

Table (3.1): Demographic characteristics among studied groups according to the infertility causes.

Variables	Cause of infertility								P value
	Unexplained infertility (5)		Male factor (19)		Female factor (5)		Combined factor (8)		
	Mean	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	
Age (year)	29.6	4.09	29.6	5	32.4	5.6	31.6	5.06	0.131
BMI (Kg/m^2)	25.5	4.49	27.50	5.33	31.3	4.36	31.6	4.24	0.079
Duration of infertility (year)	11.2	2.94	10.2	5.58	11	4.18	8.3	4.83	0.156
	Count	**%**	**Count**	**%**	**Count**	**%**	**count**	%	
Primary Infertility	3	13.6	9	40.9	4	18.2	6	27.3	0.121
Secondary Infertility	2	13.5	10	66.7	1	6.7	2	13.3	

Chi-square test, ANOVA test (LSD test), and SD: Standard deviation

3.2. Mean Hormonal Levels in the Study Groups:

The mean serum level of follicle stimulating hormone (FSH) in unexplained infertility cases was 4.62 mlU/ml and in male factor 4.45 mlU/ml, while in female factor was 7.64 mlU/ml and in combined factor 7.24 mlU/ml. However, female and combined factor were significantly different from unexplained and male factor ($P=0.000$). In addition, the mean serum level of luteinizing hormone (LH) in unexplained infertility group was 3.28 mlU/ml, in male factor group was 3.29 mlU/ml and in female

factor group was 8.16 mlU/ml, while in combined factor group was 6.26 mlU/ml. However, female factor cause was significantly different from unexplained, male and combined factor groups with (*P=0.000*).

On the other hand, the mean of progesterone in unexplained infertility was 0.78 ng/ml, in male factor was 1.3 ng/ml and in female factor was 0.93 ng/ml, while in combined factor was 0.98 ng/ml . There was no significant differences in serum levels of progesterone between patients groups (*P=0.467*). Regarding Estradiol hormone (E2) at cycle day two, the mean in the mean in unexplained infertility was 48.8 pg/ml, in male factor was 45.66 pg/ml and in female factor was 32.30 pg/ml, while in combined factor was 33.61pg/ml. However, unexplained factor was significantly different from male, female and combined factor groups with (*P=0.007*).

The anti mullerian hormone (AMH) mean in unexplained infertility was 2.91 ng/ml, in male factor was 3.30 ng/ml and in female factor group was 0.53 ng/ml, while in combined factor was 0.85 ng/ml. However, male causes was significantly different from unexplained, female and combined factors (*P=0.000).* About the mean of Estradiol hormone (E2) at day of HCG injection the mean in unexplained infertility was 2185.04 pg/ml, in male factor was 2703.85 pg/ml and in female factor was 1053.81pg/ml, while in combined factor was 1365.00 pg/ml. However, male factor was significantly different from unexplained, female and combined factor groups with (*P=0.003*).

Table (3.2) shows the mean hormone levels in the groups under study.

Variables	Cause of infertility								*P value*
	Unexplained infertility (5)		Male factor (19)		Female factor (5)		Combined factor (8)		
	Mean	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	
FSH (mIU/ml)	4.62	0.80	4.45	1.29	7.64	0.75	7.24	2.00	**0.000***
(mIU/ml)	3.28	0.38	3.29	0.70	8.16	1.78	6.26	1.78	**0.000***
Progesteron (ng/ml)	0.78	0.19	1.3	1.98	0.93	0.27	0.98	0.35	0.467
E2 day 2 (pg/ml)	48.8	7.30	45.66	5.03	32.30	5.16	33.61	4.54	**0.005***
AMH (ng/ml)	2.91	0.97	3.30	0.64	0.53	0.20	0.85	0.40	**0.000***
E2 HCG (pg/ml)	2185.04	480.77	2703.85	389.97	1053.81	823.43	1365.00	475.25	**0.003***

FSH: Follicle stimulating hormone; LH: Luteinizing hormone. E2 HCG: Estradiol Hormone following Human Chorionic Gonadotropin injection; E2 at day 2: Estradiol hormone on cycle day two; AMH: Anti-Mullerian Hormone. The standard deviation in the ANOVA (LSD test) is denoted by SD.

3.3. Clinical characteristics of different infertility groups.

The mean number of attempts of Intracytoplasmic sperm injection (ICSI) in unexplained infertility group was 1.8, in male factor cases was 1.6, in female factor group was 1.8 and in combined factor group was 1.5. no significant difference between groups (*P=0.276*). Was seen the mean of number total oocyte in unexplained infertility group was 13.40, in male factor cases was 13.94, in female factor group was 10.20, and in combined factor group was 11.71. However, unexplained infertility and male factor were significantly different from female and combined factor groups with (*P=0.056*).

Regarding the mean of Maturity rate in unexplained infertility group 88.46, in male factor cases was 79.22, in female factor group was 70.86 and in combined factor group was 80.71. There was no significant difference detected (*P=0.197*). The mean of Fertilization rate in unexplained infertility group was 86.7, in male factor cases was 78.84, in female factor group was 70.66 and in combined factor group was 64.24. However, unexplained factor was significantly different from male, female and combined factor groups with (*P=0.037*).

Also, the mean of embryo Gradel (GI) in unexplained infertility group was 3.66, in male factor cases was 4.07, in female factor group was 1.66 and in combined group was 2.00. However, male factor was significantly different from unexplained, female and combined factor groups with (*P= 0.001*). In addition, the mean of embryo Grade ll (GII) in unexplained infertility group was 3.66, in male factor cases was 3.57, in female factor group was 2.33 and in combined factor group was 2.00. However, unexplained and male factor groups were significantly different from female and combined factor groups (*P =0.059*). About the mean of embryo Grade III (GIII) in unexplained infertility group was 3.00, in male factor cases was 2.42, in female factor group was 2.33 and in combined factor group was 2.50. However, no significant difference between groups (*P=0.488*). Concerning the mean of Transferred embryo in unexplained infertility group was 3.20, in male factor cases was 3.50, in female factor group was 2.40 and in combined factor group was 2.83. However, unexplained and male factor was significantly different from female and combined factor groups with (*P= 0.057*).

The highest pregnancy rate was identified within the male factor infertility group, where 52.63 % of the female showed successful pregnancy as shown by positive result of serum B HCG. On the other hand, the lowest

pregnancy rate (20%) was seen when the cause of infertility was a female factor.

Table (3.3): Clinical characteristics of different infertility groups

Variables	Cause of infertility								*P value*
	Unexplained infertility (5)		Male factor (19)		Female factor (5)		Combined factor (8)		
	Mean	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	
ICSI attempt	1.8	1.09	1.6	0.67	1.8	1.09	1.5	0.75	0.276
Total oocytes	13.40	6.87	13.94	5.86	10.20	7.85	11.71	4.99	**0.056 ***
Maturity rate	88.46	12.09	79.22	21.24	70.86	23.73	80.71	15.76	0.197
Fertilization rate	86.7	12.16	78.84	17.41	70.66	40.44	64.24	29.80	**0.037 ***
Embryo GI	3.66	2.88	4.07	2.01	1.66	0.57	2.00	0.81	**0.001 ***
Embryo GII	3.66	2.30	3.57	2.10	2.33	0.57	2.00	1.41	**0.059 ***
Embryo GIII	3.00	2.64	2.42	1.74	2.33	1.15	2.50	1.73	0.488
Transferred embryo	3.20	0.44	3.50	0.78	2.40	1.14	2.83	1.16	**0.057 ***
	Count	**%**	**Count**	**%**	**Count**	**%**	**Count**	**%**	

B HCG result -ve	3	60	9	47.37	4	80	6	75	0.121
+ve	2	40	10	52.63	1	20	2	25	

B-HCG (beta-human chorionic gonadotropins), GI (grade L), ICSI (intracytoplasmic sperm injection), ANOVA (LSD test), Chi-square test, and (*): significant at $p<0.05$.

3.4. Cytokine levels among studied groups

The mean of IL-6 level in the serum of unexplained infertility cases was 115.50. while in male, female and combined factors were 197.63, 376.55 and 298.15 respectively. There was no significant difference between groups (*P= 0.245*). Whereas, the mean of IL-6 follicular fluid level of unexplained infertility was 64.40, in male factor 51.41, in female factor 66.75 and in combined factor 78.7. There was no significant difference between groups (*P=0.239*), as found in table (3-4).

Regarding the mean of IL-10 level in the serum of unexplained infertility cases was 79.27, while, in male, female and combined factors were 95.23, 71.50 and 82.42 respectively. However, no significant difference between groups (*P=0.454*). Whereas, the mean of IL-10 follicular fluid level of unexplained infertility was 37.3, in male factor 57.1, in female factor 29.4 and in combined factor 51.9. However, no significant difference between groups (*P=0.497*).

On the other hand, the mean of EG-VEGF level in serum of unexplained infertility cases was 813.0, while, in male, female and combined factor were 1266, 1276.6 and 960.8 respectively. There was no significant difference between groups (*P=0.494*). Whereas, the mean of EG-VEGF level in follicular fluid of unexplained factor group 1529, in male factor

1537.8, in female factor 1538 and in combined factor 994.2 .There were no significant difference between groups (*P=0.283*).

Table (3.4) shows the levels of cytokines in the groups under study.

Interleukins	Causes of infertility								*P value*
	Unexplained factor (5)		Male factor (19)		Female factor (5)		Combined factor (8)		
	Mean	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	
IL-6 in serum	115.50	30.05	197.63	373.58	376.55	803.2	298.15	563.25	0.245
IL-6 in follicular fluid	64.40	48.22	51.14	62.56	66.75	48.17	78.7	83.7	0.239
IL-10 in serum	79.27	97.33	95.23	149.48	71.50	16.26	82.42	82.83	0.454
IL-10 in follicular fluid	37.3	50.5	57.1	102.7	29.4	41.8	51.9	39.09	0.497
EG-VEGF in serum	813.0	560.3	1266	2285	1276.6	1210.7	960.8	962.8	0.494
EG-VEGF in follicular fluid	1529	1537.7	1537.8	2183.1	1538	1669	994.2	1000	0.283

IL: Interleukin, ANOVA test (LSD test)

3.5. Mean differences in demographic characteristics based on B HCG (pregnancy results)

Regarding pregnant women, the mean of age, body max index and duration of infertility were 30.26, 25.65 and 11.0 respectively, while, in non-pregnant women the mean of age, body max index and duration of infertility were 30.54, 31.2 and 9.409 respectively. There was no significant difference

between B HCG result groups with age and duration of infertility (*P=0.434*, *P=0.170)*. While, the max index in non-pregnant women was significantly different from pregnant women with *(p=0.000),* as clarified in table (3-5).

About the types of infertility, the count of primary infertility in pregnant women was 2 and 20 in non-pregnant women while the count of secondary infertility in pregnant women 13 and 2 in non-pregnant women. There was significant relation to the B HCG result (*P=0.000*).

Concerning the infertility causes in pregnant women, the count of unexplained infertility, male factor, female factor and combined factor were 2, 10, 1 and 2 respectively, while, in non-pregnant women the count of unexplained infertility, male factor, female factor and combined factor were 3, 9, 4 and 6 respectively. There was no significant relation to the B-HCG result (*P=0.121*).

Table (3-5): demographic characteristics based on the B HCG (pregnancy outcome) result

Variables		B HCG				*P value*
		Positive (pregnant)		Negative (non pregnant)		
		Mean	**±SD**	**Mean**	**±SD**	
Age (year)		30.26	4.6	30.54	5.2	0.434
BMI (Kg/m^2)		25.65	3.72	31.2	4.66	**0.00***
Duration (year) of infertility		11.0	5.5	9.409	4.43	0.170
		Count	**%**	**Count**	**%**	
Type of Infertility	Primary	2	9.1	20	90.9	**0.00***
	Secondary	13	86.7	2	13.3	
Cause of infertility **Unexplained infertility**		2	40.0	3	60.0	0.121

Male factor	10	52.6	9	47.4	
Female factor	1	20.0	4	80.0	
Combined factor	2	25.0	6	75.0	

BMI (Body Mass Index) was analyzed using Student's t-test and Chi-square test, with (*) indicating significance at $p<0.05$.

3.6. Hormonal study according to the result of B HCG (Pregnancy result)

In pregnant women, the means of FSH, LH, Progesterone, E2 day 2, AMH and E2 HCG were 4.86, 4.77, 0.90, 64.84, 3.45 and 2515.1 respectively. Whereas, in non-pregnant women the means of FSH, LH, Progesterone, E2 day 2, AMH and E2 HCG were 7.39, 4.73, 0.75, 57.18, 5.39 and 2079 respectively. There was no significant difference between BHCG result groups with (p=0.098, 0.488, 0.069, 0.295, 0.268 and 0.124) respectively, as demonstrated in table (3-6).

Table (3.6): Mean differences of hormonal study according to the result of B HCG (pregnancy result)

	Positive (pregnant)		Negation(pregnant)		
	Mean	**SD**	**Mean**	**SD**	
FSH (mIU/ml)	4.86	1.46	7.39	7.18	0.098
LH (mIU/ml)	4.77	1.75	4.73	2.27	0.488
Progesterone (ng/ml)	0.90	0.41	0.75	0.29	0.069
E2 day 2 (pg/ml)	64.84	25.29	57.18	26.84	0.295
AMH (ng/ml)	3.45	2.84	5.39	10.79	0.268
E2 HCG (pg/ml)	2515.1	986.1	2079	743.5	0.124

Student’s t-test.

3.7. Clinical characteristics according to the result of B-HCG (Pregnancy result)

Current study found in pregnant women, the mean of ICSI attempts was 2.0 and in non-pregnant women the mean of ICSI attempts was 1.0.

However, pregnant women were significantly different from non-pregnant women with (*P=0.00*). While the mean of total oocyte number, maturity rate, fertilization rate, embryo GI, embryo GII, embryo GIII and transferred embryo in pregnant women were 14.46, 77.24, 76.44, 3.90, 3.46, 3.10 and 3.6 respectively and in non-pregnant women the mean of total oocyte number, Maturity rate, Fertilization rate, Embryo GI, Embryo GII, Embryo GIII and Transferred embryo were 11.70, 81.45, 75.46, 4.46, 3.37, 2.53 and 2.9 respectively. There was no significant difference between groups with (*P=0.083, 0.276, 0.453, 0.411, 0.404, 0.067 and 0.313*) respectively, as clarified in table (3-7).

Table (3.7): Mean differences of clinical characteristics according to the result of B HCG (pregnancy result)

Variables	B HCG				*P value*
	Positive (pregnant)		Negative (non pregnant)		
	Mean	**±SD**	**Mean**	**±SD**	
ICSI attempt	2.0	0.0	1.0	0.00	**0.00***
Total oocyte number	14.46	6.67	11.70	5.37	0.083
Maturity rate	77.24	20.67	81.45	18.70	0.276
Fertilization rate	76.44	17.86	75.46	28.07	0.453
Embryo GI	3.90	2.80	4.46	2.36	0.411
Embryo GII	3.46	2.16	3.37	2.06	0.404
Embryo GIII	3.10	1.72	2.53	1.45	0.067
Transferred embryo	3.6	0.89	2.9	0.83	0.313

(*) Student's t-test, significant at $p<0.05$.

3.8. Cytokines based on B HCG (pregnancy result)

Regarding the means serum and follicular concentrations of cytokines distributed according to the outcome of ICSI (succussed versus failed). The

mean serum concentration of IL-6 in serum was higher in succussed ICSI (310.71 pg/mL (±571.6)) than its mean in difference in the mea the sera in failed ICSI women (109.38(±240.6) pg/mL). This n serum IL-6 concentration was of marginal statistical significance (p=0.059). A similar trend was found with mean concentration of IL-6 levels in follicular fluid where the mean in follicular fluid of women with succussed ICSI was much higher than the mean in the follicular fluid of women with failed ICSI (156.9(±75.81) and 60.4(±13.46), respectively. However, this difference in the mean IL-6 follicular fluid levels showed marginal statistical significance (p= 0.067).

Regarding serum and Follicular fluid levels of IL-10, there were almost similar levels of this cytokine in both succussed and failed women, and consequently no significant differences were found in both serum and follicular fluid (p=0.286 and p= 0.907, respectively).

Additionally, pregnant women's follicular fluid EG-VEGF mean was 671.5, whereas non-pregnant women's was 32.2. However, there was a significant difference (P=0.041) between pregnant and non-pregnant women.

However, the mean blood level of EG-VEGF was 1496.5 in non-pregnant women and 60.96 in pregnant women. Nevertheless, there was no discernible difference between the B HCG groups (P=0.143).

Table (3.8): Mean differences of cytokines based on B HCG (pregnancy result)

Interleukins	B HCG				*P value*
	Positive (pregnant)		Negative (non-pregnant)		
	Mean	**±SD**	**Mean**	**±SD**	
IL-6 (pg/ml) in serum	310.71	571.6	109.38	240.6	**0.059***
IL-6 (pg/ml) in follicular fluid	156.96	75.81	60.15	13.46	0.067
IL-10 (pg/ml) in serum	77.07	155.9	61.01	86.11	0.286
IL-10 (pg/ml) in follicular fluid	47.3	79.8	50.6	79.5	0.907
EG-VEGF (pg/ml) in serum	609.6	666.7	1496.5	463.4	0.143
EG-VEGF (pg/ml) in follicular fluid	671.5	658.7	1932.2	2124.72	**0.041***

(*): significant at p<0.05

3.9. Correlation of study cytokines in patients

3.9.1. Correlation between study follicular cytokines with BMI and clinical characteristics

About table (3-9), there was no significant correlation between follicular cytokines with BMI and clinical characteristics (Maturity rate, Fertilization rate, Embryo GI, Embryo GII and Embryo GIII) with (*P>0.05*) except a positive correlation between EG-VEGF in Follicular fluid with embryo grade II (*P= 0.056)*.

Table (3.9): The correlation between follicular cytokines with BMI and clinical characteristics in patients

Variables		IL 6 FF	IL 10 FF	EG-VEGF FF
BMI	R	-0.006	-0.050	-0.066
	P	0.488	0.387	0.352
	N	33	35	36
Maturity rate	R	-0.145	0.077	0.095
	P	0.211	0.330	0.291
	N	33	35	36
Fertilization rate	R	-0.045	-0.025	0.245
	P	0.404	0.445	0.082
	N	32	33	34
Embryo GI	R	-0.148	0.322	0.127
	P	0.231	0.067	0.256
	N	27	28	29
Embryo GII	R	0.151	-0.135	**0.367**
	P	0.268	0.285	**0.056***
	N	19	20	**20**
Embryo GIII	R	0.193	0.313	0.039
	P	0.168	0.069	0.420
	N	27	29	29

N: Number; P: P value; r: Pearson Correlation

3.9.2. Correlation between study serum cytokines with BMI and clinical characteristics

There was no significant correlation between serum cytokines with BMI and clinical characteristics (Maturity rate, Fertilization rate, Embryo GI, Embryo GII and Embryo GIII) with (*P>0.05*) except a positive correlation between EG-VEGF serum level with embryo GII (*P= 0.049*), as in table (3-10).

Table (3.10): The correlation between serum cytokines with BMI and clinical characteristics

Variables		IL 6 S	IL 10 S	EG-VEGF S
BMI	R	-0.065	-0.066	-0.078
	P	0.357	0.363	0.325
	N	34	31	36
Maturity rate	R	-0.165	-0.108	0.123
	P	0.175	0.282	0.237
	N	34	31	36
Fertilization rate	R	0.225	0.116	-0.011
	P	0.108	0.275	0.475
	N	32	29	34
Embryo GI	R	0.129	-0.319	0.152
	P	0.260	0.060	0.216
	N	27	25	29
Embryo GII	R	-0.125	-0.178	**0.381***
	P	0.305	0.233	**0.049***
	N	19	19	**20**
Embryo GIII	R	0.092	-0.156	-0.085
	P	0.327	0.229	0.330
	N	26	25	29

P: P value, N: Number, r: Pearson Correlation, and (*): significant at p<0.05

3.10. Correlation between study serum and follicular cytokines

There was no significant correlation between serum cytokines and follicular cytokines (*P>0.05*), except between IL 6 S and IL 6 F there is a positive correlation with (*P=0.010*); IL 6 S and IL 10 S there is a positive correlation with (*P=0.000*), IL 10 S and IL 6 F there is a positive correlation with (*P=0.000*); EG-VEGF S and EG-VEGF F there is a positive correlation with (*P=0.000*), as in table (3-11).

Table (3.11): The correlation between serum and follicular cytokines

Variables		IL 6 S (pg/ml)	IL 10 S (pg/ml)	EG-VEGF S (pg/ml)
IL-6 F (pg/ml)	R	**0.385***	**0.625****	0.225
	P	**0.010**	**0.000**	0.094
	N	**36**	**36**	36
IL-10 F (pg/ml)	R	0.228	0.193	-0.129
	P	0.094	0.134	0.231
	N	35	35	35
EG-VEGF F (pg/ml)	R	-0.004	0.035	**0.683****
	P	0.491	0.420	**0.000**
	N	36	36	**36**
IL-6 S (pg/ml)	R	1	**0.632****	0.065
	P		**0.000**	.353
	N	37	**37**	36
IL-10 S(pg/ml)	R	**0.632****	1	0.134
	P	**0.000**		0.218
	N	**37**	37	36
EG-VEGF S (pg/ml)	R	0.065	0.134	1
	P	0.353	0.218	
	N	36	36	36

P: P value, N: Number, r: Pearson Correlation, and (*): significant at $p<0.05$

Chapter Four

Discussion

4. Discussion

4.1. Demographic characteristics among studied groups according to the infertility causes:

The age of patients ranged from 20-41 years with mean ages of women with unexplained infertility, male factor, female factor and combined factor were 29.6, 29.6, 32.4, 31.6 years, respectively. There were no significant differences between the patient groups with (*P =0.*131) regarding the age. This results were consistent with other studies such as the study accomplished by (Sarapik, Velthut et al. 2012) who found the mean age of male factor and female factor were (32.6), (34.8) respectively.

In addition, The mean of body max index (BMI) in patients with unexplained infertility, male factor, female and combined factor infertility (25.5, 27.5, 31.3 and 31.6 respectively). There were no significant differences in BMIs between patients groups with (P=0.079). The results of current study were related with other studies such as the study by (Spanou, Kalogiannis et al. 2018, Martinez, Baccarelli et al. 2019) whose reported the BMI means were (23.4), (23.6) of male factor and PCOS respectively with no significant association was observed (P > 0.05). Also, a study by (Mehta, Kamdar et al. 2013) in which BMI mean of male factor was (23.97) with no significant result and the study by (Kudsy, Alhalabi et al. 2016) in which BMI mean was (27.19) in PCOS group with no significant difference (*P>0.05*).

About duration of infertility, the mean values of unexplained infertility, male factor, female factor and combined factor were 11.2, 10.2, 11 and 8.3 years respectively with a non-significant difference (P= 0.156). It is consistent with other studies such as the study by (R.A, Y. et al. 2018) In which the mean no significant result. Also, the study by (Swadi, Edan et al. 2023) in which mean of infertility duration was (5) with no significant result. While

in the study by (Vural, Vural et al. 2015) in which the mean of infertility duration was (7.6) with highly significantly association (*P =0.01*) .

Regarding the types of infertility, there was no significant differences about infertility types between patient groups with (*P =0.121*) in current study. Several studies such as the study by (Swadi, Edan et al. 2023) whose reported that primary infertility count was 37 (74%) and secondary infertility was 13 (38%) of male factor infertility. The study by (R.A, Y. et al. 2018) whose found the primary infertility was (34) and secondary infertility was (11) with no significant difference.

4.2. Mean Hormonal levels in studied groups:

The mean serum level of follicle stimulating hormone (FSH) in female with unexplained infertility cases was 4.62 mlU/ml and in male factor 4.45 mlU/ml, while in female factor was 7.64 mlU/ml and in combined factor 7.24 mlU/ml. However, female and combined factor were significantly different from unexplained and male factor (*P=0.000*).

The mean of follicle stimulating hormone (FSH) in male factor was not significant, it is consistent with other studies such as the study by (Sarapik, Velthut et al. 2012) who reported the mean of FSH in male factor was (5.6), in PCOS group was (5.7), with no significant result, the levels of FSH was normal in these groups. The mean of follicle stimulating hormone (FSH) in female factor group was (7.64). female factor was significantly different from male and unexplained causes (*P=0.000*). (Rebar 2007) revealed that high levels of FSH are necessary for POR diagnosis. Present study result was consistent with other studies such as the study by (Barbakadze, Kristesashvili et al. 2015) who reported the mean of FSH were (8.96) in group (<35years) and (11.23) in group (35-40 years). There was a positive correlation between age and FSH, with a highly significant (*P<0.0001*) positive correlation with FSH level.

About the mean serum level of luteinizing hormone (LH) in unexplained infertility group was 3.28 mlU/ml, in male factor group was 3.29 mlU/ml and in female factor group was 8.16 mlU/ml, while in combined factor group was 6.26 mlU/ml. However, female factor cause was significantly different from unexplained, male and combined factor groups with (*P=0.000*).

Other studies by (Liu, Liu et al. 2019) who found the mean of LH was (3.2) and (3.8). respectively with no significant result (*P =0.20*). Regarding the female factor group mean was higher than male factor this consistent with many studies such as the study by (Lisi, Rinaldi et al. 2005) who found the endocrinological disorder which is linked to hypersecretion of LH and ovulatory dysfunction is attributed to increased levels of LH and the study by (Jain, Malik et al. 2022) revealed that mean of LH in PCO was (6.95). While, current study disagree with the study conducted by (Rawdhah, H.K.K.M. et al. 2023) whose reported the Mean of (LH) in PCOS cases was (5.46) and in healthy control was (4.53). However, the association was nonsignificant (*P*=0.429) between PCOS patients and the control. Female factor patients result agree with many studies such as the study by (Broekmans, Verweij et al. 2014) who found that previous model for the prediction of ovarian response in antagonist cycles showed high levels of LH could predict the ovarian response, and the study by (Tsakos, Tolikas et al. 2014) was found that basal LH levels to be good predictors for ovarian stimulation. While, a study by (Liu, Zhang et al. 2020) reported that mean of LH in poor ovarian reserve was 5.64 and control was 5.63 with, no significant result (*P=927*).

On the other hand, the mean of progesterone in unexplained infertility was 0.78 ng/ml, in male factor was 1.3 ng/ml and in female factor was 0.93 ng/ml, while in combined factor was 0.98 ng/ml. There were no significant differences in serum levels of progesterone between patients' groups

(*P=0.467*). However, the results in this study was different from the study of (Sahin, Madendag et al. 2020) who found the serum progesterone levels were significantly lower in unexplained infertility group than in the fertile control group (p=0.02) . the reason for this different could be due to differences in sample size.

Regarding Estradiol hormone (E2) at cycle day two, the mean in unexplained infertility was 48.8 pg/ml, in male factor was 45.66 pg/ml and in female factor was 32.30 pg/ml, while in combined factor was 33.61pg/ml. However, unexplained factor was significantly different from male, female and combined factor groups with (*P=0.007*).

It is consistent with other studies such as the study by (Zhang, Tian et al. 2019) who found the mean of Estradiol hormone in non PCO cases was (53.8) and in the PCOS cases was (51.97) with no significant result. Concerning Estradiol hormone (E2) at cycle day two in female factor patients, several studies conducted by (Liu, Zhang et al. 2020) and (Zhang, Zhang et al. 2021) whose revealed that mean of Estradiol hormone were (30.10) and (34.8) respectively with result.

Regarding the anti-Mullerian hormone (AMH) mean in unexplained infertility was 2.91 ng/ml, in male factor was 3.30 ng/ml and in female factor group was 0.53 ng/ml, while in combined factor was 0.85 ng/ml. However, male causes were significantly different from unexplained, female and combined factors (*P=0.000).* It is consistent with other studies such as the study by (Jain, Malik et al. 2022) They discovered that PCOS patients had an average AMH of 7.04. Another study carried out by (Liu, Zhang et al. 2020) revealed that there was a significant difference between the groups (P=0.001), with the AMH mean for POR patients being 0.58 and the control being 2.56. Furthermore, the study conducted by (Zhang, Zhang et al. 2021) There was a significant difference between young and old POR patients, and

the mean AMH in POR was shown to be (0.6) associated with the female's age. The AMH was lower in the advanced age group. Nevertheless, the research carried out by (Barbakadze, Kristesashvili et al. 2015) Reported the mean of AMH was (2.5) in group (<35years) and (1.1) in group (35-40 years) AMH values reflected age-specific changes better than other indicators.

About the mean of Estradiol hormone (E2) at day of HCG injection in unexplained infertility was 2185.04 pg/ml, in male factor was 2703.85 pg/ml and in female factor was 1053.81pg/ml, while in combined factor was 1365.00 pg/ml. However, male factor was significantly different from unexplained, female and combined factor groups with (*P=0.003*). It is similar to the study by (Kavrut, Kahraman et al. 2022) who revealed that mean of E2 on HCG day was (684.66) in POR cases.

4.3. Clinical characteristics of different infertility groups

Present study reported that the mean number of attempts of intracytoplasmic sperm injection (ICSI) in unexplained infertility group was 1.8, in male factor cases was 1.6, in female factor group was 1.8 and in combined factor group was 1.5. no significant difference between groups (*P=0.276*).

About the mean of Total oocyte number in females who had unexplained infertility and male factor (13.40,13.94) respectively, while the mean of total number of eggs decreased in females who female or combined infertility (10.20, 11.71) respectively. These differences showed marginal statistical significance (p=0.056). A study conducted by (Swadi, Edan et al. 2023) whose reported the median of total oocytes was (12) in male factor this associated with good stimulation of E2, AMH and FSH .

Regarding the mean of Maturity rate in unexplained infertility group 88.46, in male factor cases was 79.22, in female factor group was 70.86 and in combined factor group was 80.71. There was no significant difference detected (*P=0.197*). This result was inconsistent with a study of (Kamath, Mangalraj et al. 2008) who found a significant result with (*P=0.006*) between maturity rate and PCOS cause.

About fertilization rate, the mean values of unexplained infertility, male factor, female factor and combined factor in present study were 86.7, 78.84, 70.66 and 64.24 respectively. These differences showed significant result (p=0.037). This result explains the effect of cause of infertility on fertilization rate. The study showed an increase in the fertilization rate in couple who suffered from unexplained infertility and male factor, while fertilization rates decreased in women who suffered from female and combined factor. A study conducted by (Xu, Yu et al. 2022) whose reported ICSI in male infertility is significantly effective in improving the rate of normal oocyte fertilization and the clinical pregnancy rate of the cycle. It also has a low impact on adverse pregnancy outcomes and obstetric and perinatal complications and has a high safety profile. This reflects the power of ICSI to overcome male factor causes where the embryologist venally selects belt sperms for injection in addition, to normal amor female side reflecting good oocyte quality.

About the mean of embryo Gradel (GI) in unexplained infertility group was 3.66, in male factor cases was 4.07, in female factor group was 1.66 and in combined group was 2.00. These differences in grade I showed statistical significance (*P= 0.001*). In addition, the mean of embryo Grade ll (GII) in unexplained infertility group was 3.66, in male factor cases was 3.57, in female factor group was 2.33 and in combined factor group was 2.00. However, unexplained and male factor groups were significantly different

from female and combined factor groups (*P =0.059*). Current study result agreed with a study by (Sarapik, Velthut et al. 2012) He stated that the male factor was (3.8), PCO was (2.9), and POR was (1.0) for high-quality embryos (embryo Grade I and II). POR patients differed greatly from other patient populations (Sarapik, Velthut et al. 2012). Which may reflect the power of normal oocyte source to repair a wide range of abnormality in sperm, in addition to the selection of as much as passible of sperm by the embryologist.

Regarding the mean of Embryo Grade III (GIII) in unexplained infertility group was 3.00, in male factor cases was 2.42, in female factor group was 2.33 and in combined factor group was 2.50. However, no significant difference between groups (*P=0.488*). This study contradicts the study by (Lin, Yao et al. 2013) revealed to that decrease of AMH was correlated with bad quality of embryos (Embryo Grade III) .

Regarding the mean of Transferred embryo in unexplained infertility group was 3.20, in male factor cases was 3.50, in female factor cases was 2.40 and in combined factor group was 2.83. However, unexplained infertility and male factor were significantly different from female and combined factor causes (P= 0.057). Regarding female factor in present study, the low number of Oocytes and their bad quality result in a decrease number of embryos that suitable for transfer. This result compatible with a study result of (Opsahl, Blauer et al. 2001) who demonstrated the associated with number and quality of oocytes and embryos. And marked discrepancies in the size of follicles could be related to differences in follicles sensitivity to FSH and un-satisfactory maturation. This phenomenon potentially causes a decrease in the number of viable oocytes and embryos.

On the other hand, concerning the count of Beta-Human Chorionic Gonadotropins (B-HCG) test. the highest pregnancy rate after ICSI was identified with the male factor infertility group (52.63%). These results

indicate male factor has good prognostic for ICSI outcome, where's female factor is the worse. These results may explain on the basis that female factors play the most important role in pregnancy after fertilization. This result consistent with study by (Ashrafi, Sadatmahalleh et al. 2013) who reported varying ICSI success rates for different reasons of infertility and assessed the relationship between ICSI outcome and various causes of infertility. However, this study's findings differed from those of (Fhamah 2023) who found pregnancy rate not depended on quality and number of embryos but also associated with others factor effect on implantation rate. Also found no significant between B-HCG test and causes of infertility groups, this is proof that treatment for ICSI is the best option for pregnancy. When other modalities of managing failed to do so.

4.4. Cytokine levels among studied groups

The mean of IL-6 level in the serum of unexplained infertility cases was 115.50. while in male, female and combined factors were 197.63, 376.55 and 298.15 respectively. There was no significant difference between groups (*P= 0.245*). This result discrepancy to study of (Demir, Guven et al. 2009) who found the mean serum IL-6 level was significantly higher in women with unexplained infertility, compared with fertile women (5.71 and 4.31) with p=0.001 .

Whereas, the mean of IL-6 follicular fluid level of unexplained infertility was 64.40, in male factor 51.41, in female factor 66.75 and in combined factor 78.7. There was no significant difference between groups (*P=0.239*). These results were in line with (Buyalos, Funari et al. 1992) who found follicular fluid IL-6 levels were substantially higher (3 to 30-fold) than that reported in serum. There was no difference in the mean concentration of IL-6 levels between patients with antispam antibodies, endometriosis, or tubal infertility.

Regarding the mean of IL-10 level in the serum of unexplained infertility cases was 79.27, while in male, female and combined factors were 95.23, 71.50 and 82.42 respectively. However, no significant difference between groups (*P=0.454*). These result different form study of (Albayati and Abdulhameed 2024) who found significant differences in the serum levels of IL-10 between male and female factor infertility. Whereas, the mean of IL-10 follicular fluid level of unexplained infertility was 37.3, in male factor 57.1, in female factor 29.4 and in combined factor 51.9. However, no significant difference between groups (*P=0.497*). These result different form study of (Albayati and Abdulhameed 2024) who found significant differences in the serum levels of IL-10 between male and female factor infertility.

On the other hand, the mean of EG-VEGF level in serum of unexplained infertility cases was 813.0, while, in male, female and combined factor were 1266, 1276.6 and 960.8 respectively. There was no significant difference between groups (*P=0.494*). Whereas, the mean of EG-VEGF level in follicular fluid of unexplained factor group 1529, in male factor 1537.8, in female factor 1538 and in combined factor 994.2. There was no significant difference between groups (*P=0.283*). These result different form study of (Jakimiuk, Nowicka et al. 2018) who found lower levels of EG-VEGF in follicular fluid from large follicles in the group of patients with clinical pregnancy who miscarried compared to the group of patients who had no pregnancy ($p = 0.04$); when they compared this group with the group of patients who delivered a baby, there was no statistical significance. Lower levels of EG-VEGF in the follicular fluid from large follicles are negatively correlated with positive outcome of IVF, and correlate with miscarriage rates in patients after IVF. The variety of causes of infertility in the study could be behind no significant differences among the studied groups.

4.5. Demographic characteristics according to the result of B HCG (pregnancy result)

Regarding the mean of age pregnant women, the mean of age, body max index and duration of infertility were 30.26, 25.65 and 11.0 respectively, while, in non-pregnant women the mean of age, body max index and duration of infertility were 30.54, 31.2 and 9.409 respectively. There were no significant difference between B HCG result groups with age and duration of infertility (*P=0.434*, *P=0.170)*. While, the body max index was significant relation to the B HCG result *(p=0.000).*

Concerning the age, this study different with many studies such as the study by (Ahmed, Shareef et al. 2015) who found that the mean age of non-pregnant women was 33.5, whereas the mean age of pregnant women was 29.8, with a highly significant result (P=0.001). Furthermore, studies conducted by (Zahir, Al-Yasiry et al. 2020) and (Chen, Li et al. 2022) He stated that the female's age was the primary predictor of pregnancy and that if the age was raised above 35, there was a noticeable decrease in the pregnancy rate. Although this study concurred with the research by (Swadi, Edan et al. 2023) whose demonstrated the age mean of non-pregnant women was (31.80) while the age mean of pregnant women was (30.86) with no significant result (*P=0.61*). This could be attributed to that the majority of female were within the age group with better outcome reflecting the increasing attitude for not delaying this treatment option further with its bad affect in lowering the outcomes.

About BMI, this result may explain that women who have a heavy body mass have a lower pregnancy rate than women who have a lower body mass.

These results were in line with (Provost, Acharya et al. 2016) who found that increasing obesity was associated with a significant decrease in clinical pregnancy.

while, the mean of the infertility duration in our current study is consistent with the study (Swadi, Edan et al. 2023) whose reported the mean of infertility duration of non-pregnant women was (8.30) while pregnant women was (9.06)with no significant result (*P=0.63*).

About the primary and secondary types of infertility in pregnant and non-pregnant women, the highest pregnancy rate after ICSI was identified with the secondary infertility (86.7 %).

These results indicate secondary infertility has good prognostic for ICSI outcome, where's primary infertility is the worse. This result may explain the extent to which the type of infertility affects the outcome of pregnancy after IVF, since if the infertility is secondary, the success rate of pregnancy after IVF is higher.

However, the results in this study was different from the study of (Swadi, Edan et al. 2023) whose reported the primary and secondary types of infertility in pregnant women were (9) and (6) respectively, whereas, in non-pregnant women were (28) and (7) respectively with no significant result (P= 0.13) .

Concerning the infertility causes in pregnant and non-pregnant women, there were no significant association to the B HCG results (P=0.121). These results consistent with the study by (Swadi, Edan et al. 2023) whose reported there is no significant influence of infertility causes on pregnancy rate, this can be attributed to the role of ICSI in overcoming almost majority of causes of subfertility.

4.6. Hormonal study according to the result of B HCG (pregnancy result)

According to the Mean differences of FSH, LH, progesterone, E2 day 2, AMH and E2 HCG hormones in pregnant and non-pregnant women, there were no significant differences between B HCG result groups with (*P=0.*098, *P=0.*488, *P=0.069, P=0.295, P=0.268 and P=0.124*)

respectively, as demonstrated in table (3-6). This finding consistent with many studies such as the study by (Bjercke, Fedorcsak et al. 2005, Bedaiwy, Shahin et al. 2007, Al-Ghazali and Al-Jarrah 2013, Ashrafi, Sadatmahalleh et al. 2013, Cicek, Kahyaoglu et al. 2015, R.A, Y. et al. 2018) whose mentioned the same results of current study.

4.7. About the clinical characteristics according to the result of B HCG (pregnancy result)

Current study found in pregnant and non-pregnant women, there were no significant difference between groups with total oocyte number, maturity rate, fertilization rate, embryo (GI, GII and GIII) and transferred embryo (*P=0.083, P=0.276, P=0.453, P=0.411, P=0.404, P=0.067 and P= 0.313*) respectively. while concerning the ICSI attempt in pregnant and non-pregnant women, there were a significant difference with (*P=0.00*) between B HCG groups, as clarified in table (3-7).

The study showed that women who have had previous IVF have a greater possibility of becoming pregnant. This result explains that women who have had previous IVF are more careful with the doctor's instructions and take treatment regularly. Also, the doctor, through the first experience, can identify problems in the couple and avoid them in the second experience. These result were similar to (Bates Jr, Ginsburg et al. 2002) who found Women who experience an early pregnancy loss after IVF have a greater likelihood of success in subsequent IVF cycles when compared with patients who fail to conceive.

4.8. Regarding the Cytokines according to the result of B HCG (pregnancy result)

Regarding The mean serum concentration of IL-6 in serum was higher in succussed ICSI (310.71 pg/mL (±571.6)) than its mean in difference in the mean the sera in failed ICSI women (109.38(±240.6) pg/mL). This different in serum IL-6 concentration was of marginal statistical significance

(P=0.059). These results were similar to (Kim, Choi et al. 2019) who found High serum IL-6 levels in infertile women with adenomyosis can have an adverse effect on the IVF outcome including embryo quality and clinical pregnancy rate.

A similar trend was found with mean concentration of IL-6 levels in follicular fluid where the mean in follicular fluid of women with succussed ICSI was much higher than the mean in the follicular fluid of women with failed ICSI (156.96(±75.81) and 60.51(±13.46), respectively). However, this difference in the mean IL-6 follicular fluid levels showed marginal statistical significance (p= 0.067). These results were similar to (Gavrilovic, Cekovic et al. 2022) who found the mean value of IL-6 in the follicular fluid per patient was 6.59±6.29 ng/mL. The results of our study showed that there was a statistically significant difference between the 2 groups of patients that were divided first on the basis of their age (≤35 years and > 35 years), and then, according to the outcome of the IVF process (negative and positive).

Regarding serum and Follicular fluid levels of IL-10, there were almost similar levels of this cytokine in both succussed and failed women, and consequently no significant differences were found in both serum and follicular fluid (*P=0.286* and *P= 0.907*, respectively). This result different from study of (Winger and Reed 2011) who found the degree of preconception IL-10 elevation may correlate with an increased risk of IVF failure. The reason for the discrepancy between this study and study of (Winger and Reed 2011) due to differences in sample size. The number of his sample was seventy-six women who underwent IVF/ICSI cycle.

More importantly, the mean of EG-VEGF in follicular fluid, in pregnant women was (671.5) and in non-pregnant women was (1932.2). However, pregnant women were significantly different from non-pregnant women with (*P=0.041*).

While, the mean of EG-VEGF in serum, in pregnant women was (609.6) and in non-pregnant women was (1496.5). However, no significant difference between B HCG groups (*P=0.143*). as in table (3-8). These results were similar to (Jakimiuk, Nowicka et al. 2018) who found Lower levels of EG-VEGF in the follicular fluid from large follicles are negatively correlated with positive outcome of IVF, and correlate with miscarriage rates in patients after IVF. While, (Gao, Zhao et al. 2012) who found both concentrations of EG-VEGF and VEGF in FF were negatively correlated with ovarian response and oocyte maturation. Concentrations of all the three growth factors in serum were positively correlated with embryo quality, but only serum concentrations of EG-VEGF were associated with the pregnancy outcome.

4.9. Correlation of serum and FF cytokines level with BMI and clinical characteristics in patients.

In this study we found was no significant correlation between serum and follicular cytokines with BMI and clinical characteristics (Maturity rate, Fertilization rate, Embryo GI, Embryo GII and Embryo GIII) with (*P>0.05*) except a positive correlation between EG-VEGF in Follicular fluid with embryo grade II (*P= 0.056)*, and a positive correlation between EG-VEGF serum level with embryo GII (*P= 0.049*), as in table (3-10). This result similar to study of (Gao, Zhao et al. 2012) who found both concentrations of EG-VEGF and VEGF in FF were negatively correlated with ovarian response and oocyte maturation. Concentrations of all the three growth factors in serum were positively correlated with embryo quality, but only serum concentrations of EG-VEGF were associated with the pregnancy outcome

4.10. Correlation between study serum and follicular cytokines in patients

There was no significant correlation between serum cytokines and follicular cytokines (*P>0.05*), except between IL 6 S and IL 6 F there is a positive correlation with (*P=0.010*); IL 6 S and IL 10 S there is a positive correlation with (*P=0.000*), IL 10 S and IL 6 F there is a positive correlation with (*P=0.000*); EG-VEGF S and EG-VEGF F there is a positive correlation with (*P=0.000*), as in table (3-11).

Conclusions and Recommendations

Conclusions:

1. The hormonal level of FSH and LH were higher in female and combined factors with significantly different form male and unexplained factors. While, E2, AMH and E2 HCG were lows in female and combined form male and unexplained factors.
2. The total follicle number, fertilization rate, Embryo Grade l (GI), Embryo Grade II (GII), transferred embryo and pregnancy rate in unexplained and male factor high significant different from female and combined factors.
3. The lack of significant differences in IL-6, IL-10, and EG-VEGF levels across groups indicates that these cytokines may not be primary markers for distinguishing between different causes of infertile.
4. The negative correlation between BMI and IVF success underscores the importance of weight management in improving fertility outcomes
5. The positive correlation between the number of ICSI attempts and IVF success suggests that repeated attempts may increase the likelihood of a successful pregnancy.
6. IL-6 in serum high significant different in pregnant women from non-pregnant women, while EG-VEGF in follicular fluid was negative correlation with positive pregnant result.
7. Positive correlation between EG-VEGF in serum with embryo GIII and EG-VEGF in follicular fluid with embryo GII suggest that EG-VEGF may play a role in embryo development and quality.
8. Positive correlation between IL-6 and EG-VEGF in serum with their levels in follicular fluid

Recommendations:

1. Further studies with a larger samples size for each group of infertility causes are needed to better understand the impact of immunological markers.
2. Other studies are required to evaluate the association of immune markers with others hormones such as prolactin and estrogen or clinical characteristics such as antral follicle count (AFC).
3. Studying the role of immunological markers with details of the causes of female infertility, such as endometriosis, polycystic ovary syndrome and poor ovarian reserve.
4. Studying the role of these immunological markers in diseases of the female reproductive system other than infertility.
5. The effect of other cytokines on ICSI outcome need to be studied.
6. These findings highlight the complex interplay between hormonal and cytokine factors in infertility and IVF outcomes, emphasizing the need for personalized approaches in fertility treatments

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Appendix

Appendix

Appendix I: patients questionnaires

Pt. serial no.	
Pt. phone no.	
Pt. name	
Age	
BMI	
Type of infertility	
Factors(male or female)	
Cause	
Duration	
ICSI attempt	

Hormonal analysis at cycle day 2 (CD2)

1- FSH	
2- LH	
3- PROGESTERON	
4- A.M.H	
5- E2	

Table of oocyte at day of retrieval:

Total no. of oocyte retrieved	GV	Ml	Mll	Oocyte (Mll) quality	Total no. of injected (Mll)

Appendix

Table of fertilization rate at day1 :

No. Of oocytes successfully fertilization	No. Of oocytes that failed fertilization

Day2 or 3 after oocytes retrieval:

Total no. of embryos	Embryos quality (I,II,III)	Total no. of embryos transferred	Date of embryos transfer

Serum B-HCG Result: Positive or negative

Appendix II: ELISA instruments

Appendix

الخلاصة

العقم هو عدم القدرة على تحقيق الحمل بعد وقت مناسب من الجماع دون اتخاذ تدابير لمنع الحمل. تعد اضطرابات الإباضة أحد العوامل الأنثوية التي تسبب العقم، بما في ذلك تكيس المبايض وضعف احتياطي المبيض. ينتج نسيج المبيض بالإضافة إلى الخلايا المناعية السايتوكينات. توصف وظيفة السيتوكين في المبيض بأنها تعزز عمليات نمو الجريبات وتكوين الستيرويد وتجنيد وتنشيط الكريات البيضاء اللازمة للإباضة وإعادة تشكيل الأنسجة أثناء الإباضة واللوتين والتحلل الأصفر. هدفت الدراسة الحالية إلى تقييم الإنترلوكين (6)، الإنترلوكين (10) وعامل نمو بطانة الاوعيه الدمويه المشتق من الغدد الصماء البشريه في السائل الجريبي والمصل ودرست العلاقة بين هذه العلامات ونتائج حقن الحيوانات المنوية داخل السايتوبلازم .

شملت هذه الدراسة المقطعية أشخاصًا مقسمين إلى أربع مجموعات بناءً على سبب العقم: العقم غير المبرر، والعامل الذكري، والعامل الأنثوي، والعوامل المركبة. أُجريت الدراسة في مستشفى الكفيل فائق التخصص في كربلاء من فبراير 2024 إلى يونيو 2024.

تم جمع عينات المصل والسائل الجريبي في يوم استرجاع البويضة، وتم قياس مستويات IL-6 و-IL 10و EG-VEGFباستخدام تقنيةELISA

أظهرت نتيجة الدراسة الحالية أن FSH و LHكانا أعلى بشكل ملحوظ في مجموعات الإناث والعوامل المركبة مقارنة بمجموعات الذكور والعوامل غير المبررة، بينما كانت مستويات E2 في اليوم الثاني، وAMH، و E2 HCGأقل في مجموعات الإناث والعوامل المركبة مقارنة بمجموعات الذكور والعوامل غير المبررة. بالإضافة إلى ذلك، تم حساب العدد الإجمالي للبصيلات، ومعدل الإخصاب، ودرجة الجنين الأولى(GI) ، ودرجة الجنين الثانية(GII) ، وعددالأجنة المنقولة، وكان معدل الحمل في العامل غير المبرر والذكور مرتفعًا، ويختلف اختلافًا كبيرًا عن مجموعتي العامل الأنثوي والعامل المشترك.

فيما يتعلق بمستويات السيتوكينات في المصل والجريب، لم تُلاحظ فروق ذات دلالة إحصائية في مستويات IL-6 و IL-10و EG-VEGFفي السائل الجريبي والمصل عبر مجموعات المرضى.

علاوة على ذلك، كانت مستويات IL-6 في المصل أعلى بكثير لدى النساء الحوامل مقارنة بالنساء غير الحوامل. بالإضافة إلى ذلك، أظهرت مستويات EG-VEGF في السائل الجريبي ارتباطًا سلبيًا بنتائج الحمل الإيجابية.

علاوة على ذلك، لوحظت في هذه الدراسة ارتباطات إيجابية بين EG-VEGF في المصل والجنين من الدرجة الثالثة(GIII) ، وبين EG-VEGF في السائل الجريبي والجنين من الدرجة الثانية .(GII)

بالإضافة إلى ذلك، وُجدت أيضًا ارتباطات إيجابية بين مستويات IL-6 و EG-VEGFفي المصل ومستوياتهما المقابلة في السائل الجريبي.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
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تقييم الانترلوكينات 6 و 10 و EG-VEGF في وقت سحب البيوض وارتباطها بنتائج دورة التلقيح الصناعي والحقن المجهري

رسالة

مقدمة الى مجلس كلية الطب/جامعة كربلاء وهي جزء من متطلبات نيل درجة الماجستير في الاحياء المجهرية الطبية/الاحياء المجهرية

من قبل الطالبة

ايات حاتم عبد الهاشم

كلية العلوم–جامعة بابل / بكالوريوس علوم حياة

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2025 م **1446هـ**