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Department of Physiology and Medical Physics

The Effects of Serum and Follicular Fluid Thyroid Hormones on Intra-Cytoplasmic Sperm Injection (ICSI) Outcomes

A Thesis

Submitted to the College of Medicine and Committee of Postgraduate study/University of Babylon as Partial Fulfillment of the Requirements for the Degree of Master in Science / Medical Physiology.

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وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا ۝

صدق الله العلي العظيم

سورة الأسراء الآية (٨٥)

Supervisor Certificate

We certify that this thesis was prepared under our supervision at the College of Medicine/ University of Babylon, as a partial fulfillment of the requirements for the Degree of Master of Science in Medical Physiology.

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Dedication:

To all couples who deprived of children those who are suffering, hoping and waiting eagerly to look in their child eyes and touch his/her small hands .

Noor…

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Noor

Summary

Background:

The thyroid gland plays a significant role in regulating reproductive function and preserving pregnancy balance.

Thyroid dysfunction is dominant in women of childbearing age, and it has been independently linked to poor fertility and pregnancy outcomes, whether in spontaneous conception or through assisted reproductive technologies (ART).

In Vitro fertilization had been resolved a lot of infertility problems including sever ones; however, the outcomes are beyond the satisfactory goals which opened the door widely for studying any assumed factors that could be manipulated to improve them.

Aim: The current study; was aimed to examine the correlation of serum and follicular fluid thyroid hormones levels with the intra cytoplasmic sperm injection (ICSI) outcomes.

Patients, Materials and Methods: The study was designed as a prospective cohort study. It had received approval from the College of Medicine/University of Babylon's local research ethical committee.

Fifty sub fertile couples from ICSI attenders in the Fertility Center of Al-Kafeel super-specialty hospital /Karbala were involved in the study within the period from July 2021 to July 2022. Both partners had undergone evaluations from urologists and gynecologists. The assessment based on the medical history and physical examination as well as initial investigations for infertility assessment.

At the day of oocyte pick up, blood was taken and follicular fluid was collected after oocyte collection and both centrifuged then frozen at -80ºc till the time of thyroid hormone assessment by thyroid hormones ELISA Kit. While the oocyte denuded and checked under microscope for

number, maturation and morphology. The ICSI was performed and its outcomes (in form of fertilization, embryonic morphological grade and biochemical pregnancy) in correlation for thyroid hormone level was assessed later on.

Results: Serum thyroid stimulating hormone was significant in predicting pregnancy rate with p-value=0.01 and mean was relatively higher in pregnant women than non-pregnant ones (2.13 ± 0.93 vs. 1.53 ± 0.67). In addition to S.TSH had a significant correlation to follicular fluid TSH with r=0.33 and p-value=0.01. FSH level had a positive correlation with S.T4(r=0.32, p=0.02). FSH and AMH levels were found to have a positive associations with FF.T4 (r = 0.31, p = 0.02) and (r = 0.35, p = 0.01) respectively. E2 levels at day of hCG injection were found to have a strong associations with FF.T3(r=0.31 and p=0.02).

Furthermore FF.T4 and FF.T3 had a significant positive correlation with oocyte number, MII and 2PN.

Regarding to binary logistic regression S.TSH own a p-value equal to 0.00 and odds ratio =2.64

Lastly, ROC curve was done and revealed that S.TSH had higher area under the curve with cut of value of 1.5 provided a sensitivity 80% and specificity 72%.

Conclusion: Serum thyroid stimulating hormone was considered as predicting factor of pregnancy success with cut-off value equal to 1.5 and increase in S-TSH within specific limit associated with success pregnancy about three times when compared with failed group.

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List of Abbreviations

AMH	**Anti Mullerian hormone**
ART	**Assisted reproductive technique**
BMI	**Body mass index**
COC	**Cumulus Oocyte complex**
COH	**Controlled ovarian hyper stimulation**
DNA	**Deoxyribonucleic acid**
E2	**Estradiol 2**
ELISA	**Enzyme linked immunosorbent assay**
ESHRE	**European society of human reproduction and embryology**
ET	**Embryo transfer**
FF	**Follicular fluid**
FSH	**Follicle stimulating hormone**
FT3	**Free tri-iodothyronine**
FT4	**Free thyroxin**

GnRH	**Gonadotropin releasing hormone**
GV	**Germinal vesicle**
hCG	**Human chorionic gonadotropin**
HEPES	**4-(2-Hydroxyethyl)-1-piperazine ethane sulfonic acid**
ICSI	**Intra cytoplasmic sperm injection**
ICSI/ET	**Intra cytoplasmic sperm injection/Embryo transfer**
IVF	**In vitro fertilization**
LH	**Luteinizing hormone**
MI	**Meiosis I**
MII	**Meiosis II**
OHSS	**Ovarian hyper stimulation syndrome**
PN	**Pronuclei**
PT	**Pregnancy test**

PVP	**Polyvinyl pyrrolidone**
ROC	**Receiver operating characteristic**
SD	**Standard deviation**
SPSS	**Statistical package for the social science**
T3	**Tri-iodothyronine**
T4	**Thyroxine**
TBG	**Thyroxine binding globulin**
TFT	**Thyroid function test**
TESE	**Testicular sperm extraction**
THs	**Thyroid hormones**
TRH	**Thyroid releasing hormone**
TSH	**Thyroid stimulating hormone**
U/S	**Ultrasound**
WHO	**World health organization**

Chapter One

Introduction

1.1.Introduction

Infertility has established as a significant global health issue, and its incidence rate is rising yearly (Priskorn *et al.*, 2021).

Infertility can be defined as the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse or due to an impairment of a person's capacity to reproduce either as an individual or with his/her partner (Anshina *et al.*, 2019).

Infertility may become the 3rd main class of diseases in the 21st century, after tumors, and cardiovascular disorder, Its impact has been observed globally, which has a detrimental impact on family happiness and personal quality of life, but also has an endless negative effect on the level of medical services, reproductive health, social, economic as well as cultural levels in the country and the region (LIN, 2021).

Thyroid hormones (THs) may influence follicle growth by promoting granulosa cell proliferation and causing certain changes in the expression of genes involved in steroidogenesis, which is a crucial function of the thyroid gland in maintaining pregnancy stability (Huang *et al.*, 2021).

Thyroid dysfunction is dominant in women of childbearing age, and it has been independently linked to poor fertility and pregnancy outcomes, whether in spontaneous conception or through assisted reproductive technologies (ART) (Unuane and Velkeniers, 2020).

However, the exact prevalence of infertility in women with thyroid disorders remains unknown, fertility problems may persist even after restoring normal thyroid function, and then an assisted reproductive technology may be necessary to get a pregnancy (Poppe, 2021).

Intra-cytoplasmic sperm injection is one of assisted reproductive technologies (ART) that include the in-vitro handling of human oocytes,

sperm, zygotes as well as embryos, for the goal of establishing a pregnancy (Bai *et al.*, 2020).

Follicular fluid (FF): which is composed of granulosa and theca cell secretions, in addition to filtered blood plasma, is usually collected during IVF\ICSI, to be used for analysis. Follicular fluid offers a better evaluation of exposures that might affect reproductive results, as it more closely reveals the microenvironment surrounding the developing oocyte (Butts *et al.*, 2021).

Since biological effects of THs are regulated by deiodinase (DIO) in peripheral tissue, serum TH levels do not always predict tissue-specific effects in target organs, and local THs may play a direct role in physiological functions. Changes in the FF levels of hormones and metabolites have been reported to affect oocyte quality, early embryo development, and subsequent pregnancy (Cai *et al.*, 2019).

As a result of advanced technologies and the number of fertility services providing ART, the number of infants are born by ART procedures are increasing worldwide, in high-income countries, ART pregnancies represent 1.5 to 5.9% of all births ,despite the success of ART to overcome infertility, concern is growing on studying the effects of any factor that may contribute to influence conceiving success rate (Silva *et al*., 2020).

Although thyroid hormones have been studied in ICSI cycles by many researchers, however, in Iraq, the ICSI procedure is relatively new, and extensive studies in many aspects correlated with ICSI are important to be revealed regarding Iraqi women.

Additionally, due to the possible role of the thyroid hormone on ICSI outcome we proposed this study.

1.2.Aim of The Study

The purpose of this study is: to show if there is correlation of serum and follicular fluid thyroid hormones levels and the intra-cytoplasmic sperm injection (ICSI) outcomes.

Chapter Two

Review of Literature

Review of Literature

2.1. Physiologic Anatomy of the Female Reproductive Organs

The reproductive tract of female is composed of ovaries: in which the follicles develop to fully mature oocytes, the oviducts: where the fertilization occurs and the fertilized ovum(zygote) passes through it to uterus: where implantation occur, cervix and vagina: where, the fetus is passing through them and is born and all these tracts for accommodation and passage of sperms (Ali and AL-Murshidi 2018).

2.1.1. Hormonal Regulation

The hypothalamus is responsible for the pulsatile secretion of gonadotrophin-releasing hormone (GnRH) which stimulate the anterior pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH), the ovaries are response to FSH and LH for the production and periodic release of oocytes and for production of estradiol and progesterone, which prepare the endometrium for implantation (Murray and Orr, 2020).

The granulosa cells of the growing follicle release estrogen, which has a negative feedback effect on LH production during the first phase of the menstrual cycle. However, as oocytes develop in preparation for ovulation and estrogen levels reach a crucial level, estrogen starts to exert positive feedback on LH production, causing the LH surge through its effect on GnRH pulse rate (JE *et al.*, 2021).

Figure 2-1 demonstrate the regulation of hypothalamic pituitary gonadal axis

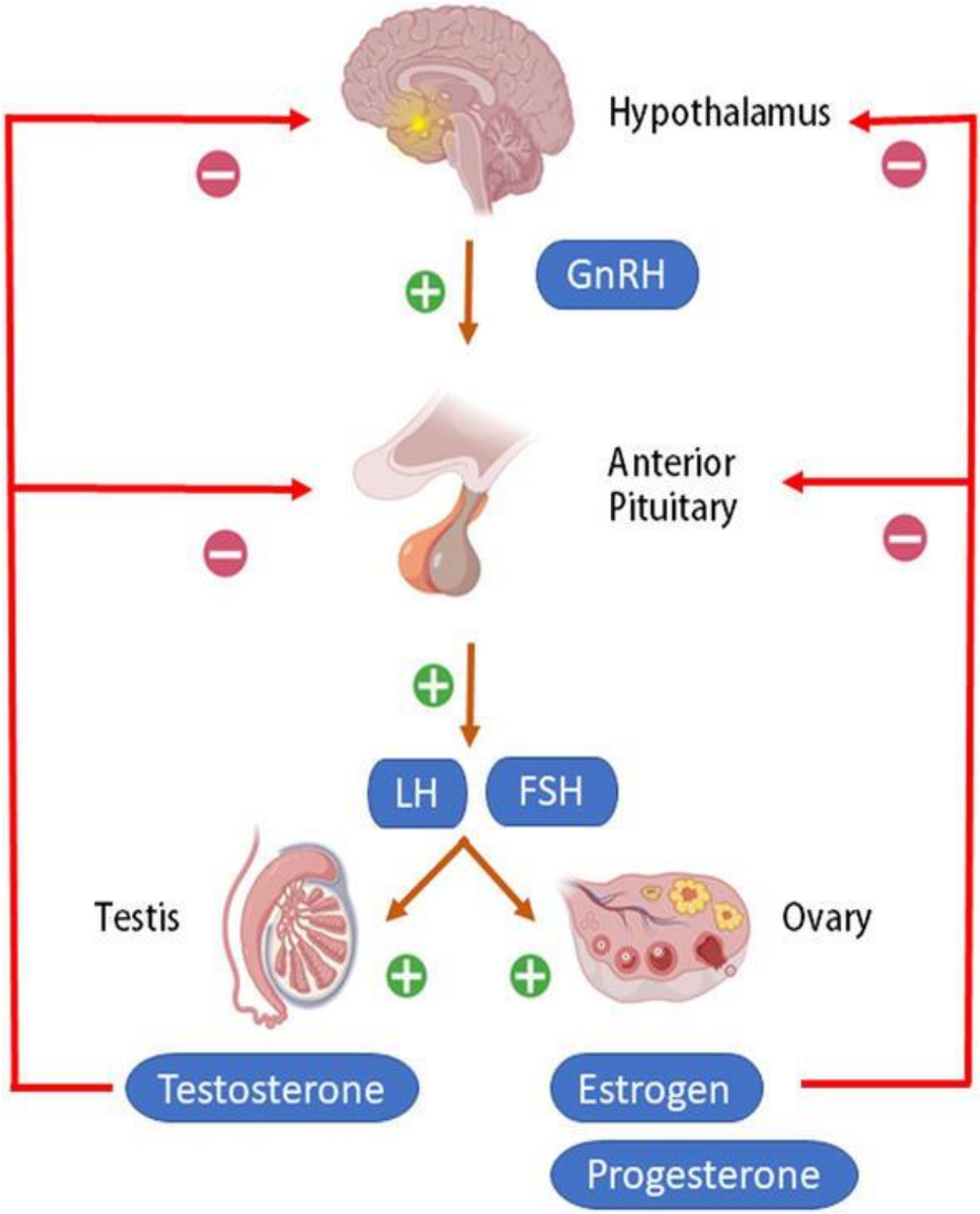

Figure 2-1. Regulation of hypothalamic pituitary gonadal axis (Gupta *et al.*, 2021).

2.1.2. Ovarian Function

The main ovarian functions are:

1. **Oogenesis**

Oogenesis is defined as the process by which the oogonia will differentiate into a mature oocyte, shortly after primordial germ cells arrival to the gonadal ridge, they are differentiated in to oogonia (Hassan, 2017).

Several oogonia have become atretic by the seventh month of prenatal development, and the only remaining oogonia cells identified as primordial oocytes go through mitosis division to produce primary oocytes (Abbara *et al.,* 2018).

After puberty and just before ovulation, the first meiotic division is completed, one of the daughter cells called the secondary oocyte, while the other, is first polar body which degenerate. The secondary oocyte immediately begins the 2nd meiotic division, but it stops at metaphase II(MII) and is completed only if a fertilization occurs, when, the 2nd polar body is discarded and the fertilized ovum started division by mitosis in a process called cleavage to produce blastomers (Ganong 2019) as in figure 2-2.

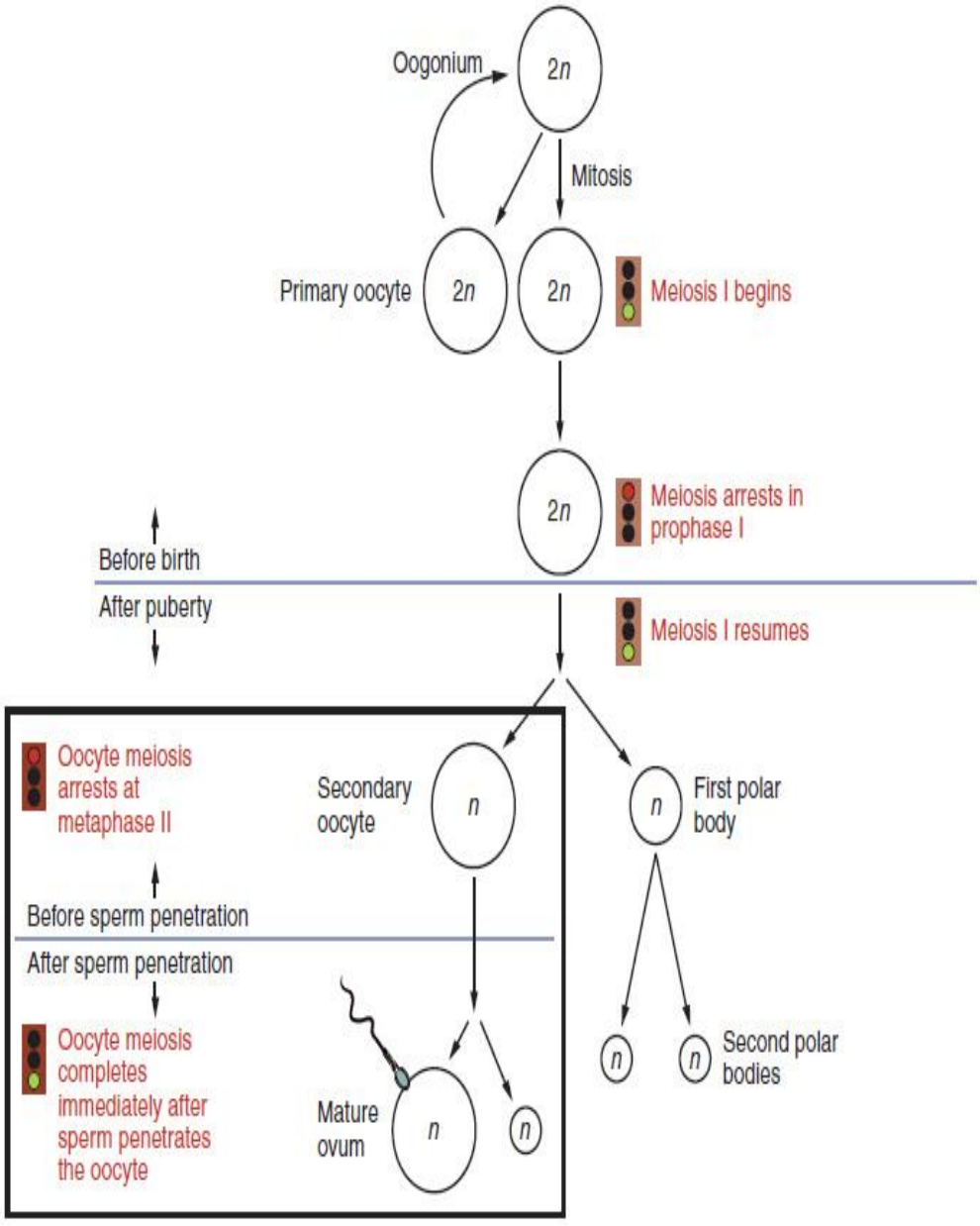

Figure 2-2. Oogenesis process; 2n: diploid nuclear material (46 chromosomes or 23 pairs), n; haploid nuclear material (23 chromosome) (Alfatlawy *et al.*, 2020).

2. Sex hormones production

Which consist of estrogen, progesterone and androgens, the main estrogenic and most potent form produced by the ovary is b-estradiol also, the ovary is responsible of production of many other peptide hormones ,such as Anti-Mullerian hormone (AMH), Inhibin A, Inhibin B, in addition to relaxin (Rosenfield *et al.*, 2021).

2.1.3. Ovarian and Endometrial Cycle

Female ovarian cycle undergo by 3 phases :

1) Follicular phase : Which starts on day 1 of menses until ovulation in which, multiple follicles grow under the effect of FSH, these growing follicles secrete estrogen, which then inhibits FSH secretion in a negative feedback mechanism including the pituitary gland, hypothalamus, in addition to inhibin B (Reed and Carr, 2018).

Usually ,every twenty eight days, gonadotropic hormones from the anterior pituitary gland promote eight to twelve new primordial follicles to begin to grow in the ovaries, one of these follicles lastly turn into "mature" and ovulates about the 14th day of the cycle (Guyton and Hall, 2019).

2) Ovulatory phase : The persistent high estrogen level induces a sudden release of LH from the pituitary gland, LH surge then triggers final maturation and ovulation through increasing collagenase activity and prostaglandin production (Kılıçdağ and Şimşek, 2020).

3) Luteal phase: The dominant follicle becomes a corpus luteum after ovulation, which secretes estrogen and progesterone. and degenerates after two weeks if pregnancy does not happen to initiate menstruation (Su *et al.*, 2017).

The proliferative phase of the endometrial cycle equal to the ovarian "follicular " phase, in which, vascular and endometrial tissues submit to extensive proliferation. After ovulation and formation of a corpus luteum, progesterone is secreted, secretory phase of the endometrial cycle that is equal to (ovarian "luteal" phase), progesterone production is essential for the establishing and maintenance of implantation and pregnancy (Critchley *et al.*, 2020).

If implantation does not happened that lead to regression of the corpus luteum (forming a scar like structure in the ovary known as the

corpus albicans) and a consequent sharp decline in circulating progesterone and estradiol concentrations, so menstruation occurs due to fall in progesterone and estrogen levels (Le *et al.*, 2020) as in figure 2-3.

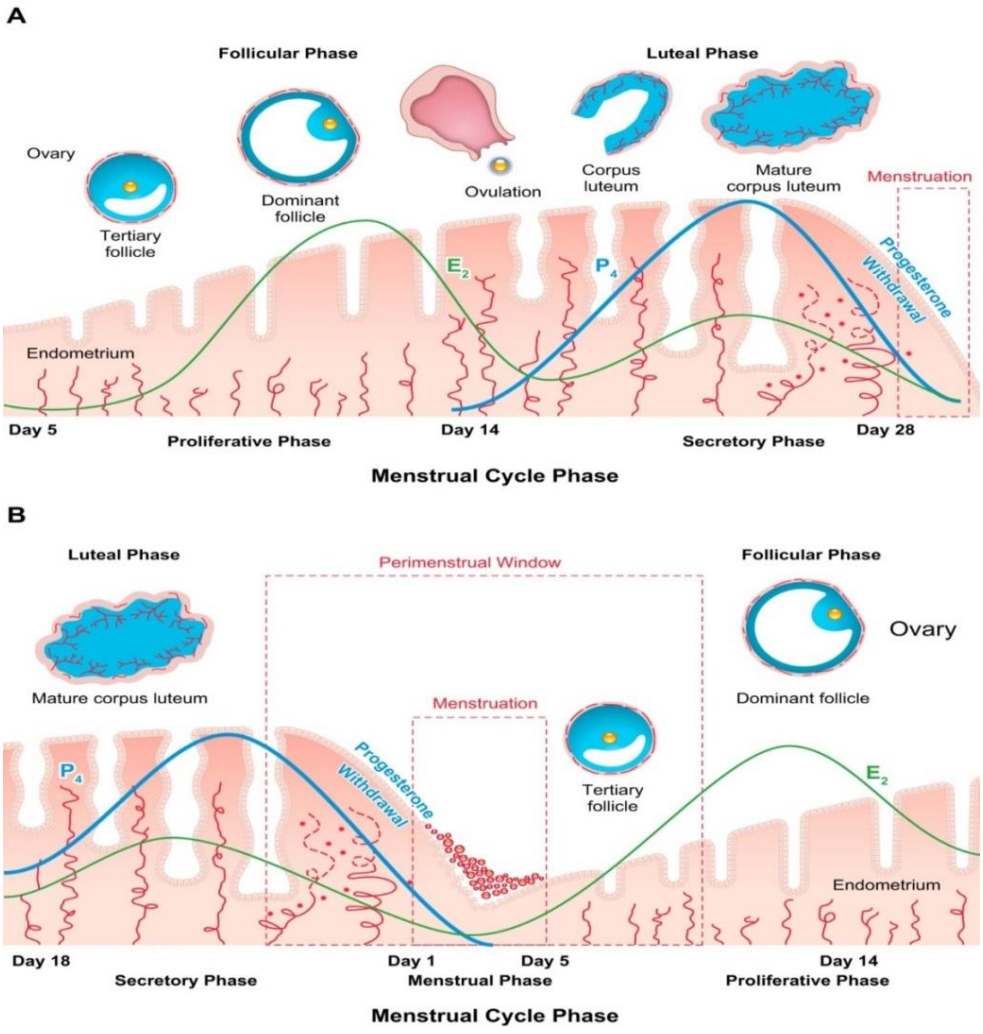

Figure 2-3. menstrual cycle. A: estradiol is the main hormone during the proliferative phase. The secretory phase take place following to ovulation. B: peri-menstrual window: focus on the significant endocrine and endometrial changes that occur through menstrual break and repair (Critchley *et al.*, 2020).

2.1.4. Follicular Fluid (FF)

The human follicular fluid (FF), which is composed of a diverse range of physiologically active chemicals, serves as the oocyte's microenvironment during its development and maturity. It is a byproduct of the blood-follicular barrier's translocation of plasma components and the secretory activity of granulosa and theca cells (Usman *et al.*, 2021).

It is usually collected during IVF\ICSI procedure and it has a greater ability to expose environment surrounding the developing oocyte, it most likely offers a more accurate assessment of exposures that might have an impact on reproductive outcomes (Butts *et al.*, 2021).

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 steroido-genesis, development of the follicles, maturation of the oocytes, ovulation, as well as their transport to the oviduct (Güngör and Güngör, 2021).

2.1.5. Ovarian Reserve

Ovarian reserve is determining the capacity of the ovary to provide egg cells which was assessed based on age, the baseline FSH level, and the baseline antral follicle count (AFC), besides baseline anti-Mullerian hormone (AMH) level. AMH is produced by granulosa cells of small, growing follicles in the ovary. Serum levels of AMH are strongly correlate with the number of growing follicles, and consequently AMH has established increasing attention as a marker for ovarian reserve (Moolhuijsen and Visser, 2020).

Beside the age which has an important role influencing egg quality and ovarian reserve. Reduced ovarian reserve describes the diminished quantity and quality of oocytes. Between 6% and 64% of infertile women

of various ages have decreased ovarian reserve. These patients also complain of increasing use of ovulation stimulants, ovarian hypo-response, and a high rate of ovulation cancellation, in addition to having fewer and lower-quality surviving oocytes (Mamsen *et al.*, 2021).

Following treatment with assisted reproductive technology (ART), these women may still experience a high rate of miscarriage, a decrease in the number of eggs obtained, and a decrease in clinical pregnancy and live births.

The physiological and psychological difficulties on these women are considerably increased by recurrent ovulation cancellation, post-ovulation fertilization failure, and failures of implantation (Chang *et al.*, 2018) as in figure 2-4.

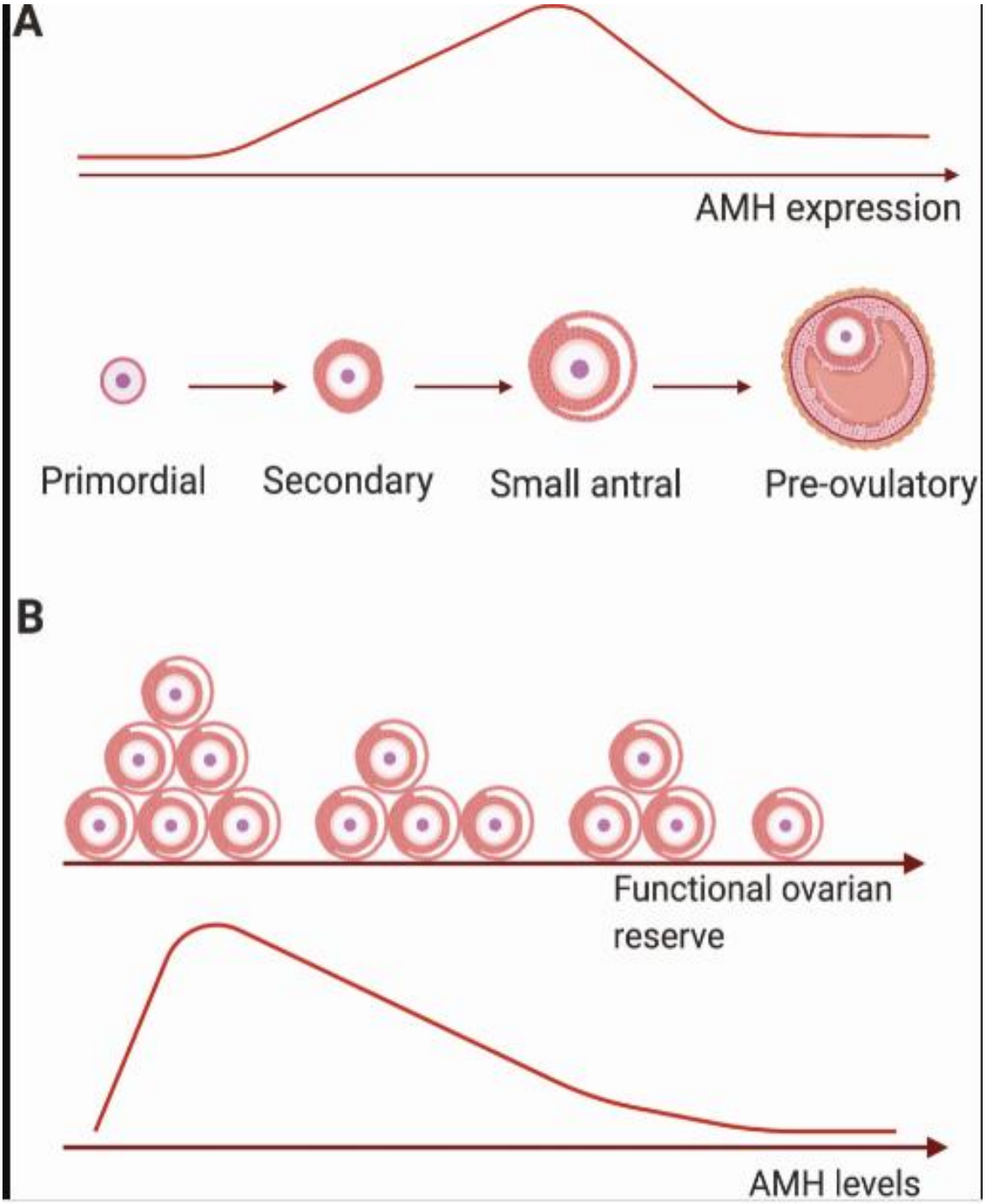

Figure 2-4. Anti-müllerian hormone level in relation to folliculogenesis and ovarian reserve. (A) From the secondary stage onward until the tiny antral follicle stage, AMH expression rises. AMH is only expressed in the cumulus granulosa cells next to the oocyte in pre-ovulatory follicles (dark pink layer). (B) Because the primordial follicle pool is depleted with age, the functional ovarian reserve decreases. As a result, there are fewer tiny antral follicles, which causes serum AMH levels to drop until they are undetectable at menopause (Moolhuijsen and Visser, 2020).

2.2. Infertility

A couple that attempts unsuccessfully to have a baby after a specific period of time is sometimes said to be sub-fertile, that mean a lesser fertile than the typical couples, infertility and subfertility are defined as the failure to achieve a clinical pregnancy after at least one year or more of regular unprotected sexual intercourse, so often two terms (infertility and sub-fertility) are overlapping (Mustafa *et al.*, 2019).
"Infertility" is not synonymous with "sterility" which is define as a permanent state of infertility (Kadir and Veleva, 2020).

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 (El Adlani *et al.*, 2021).

For decades the burden of couples' infertility has been often and disproportionately supposed as the women responsibility, as such, for biological and social causes, a couple's infertility has been unequally shared, with the tendency to investigate the female partner over the male (Pozzi *et al.*, 2021).

2.2.1. Types of Infertility (Zayed and El-Hadidy, 2020)

1) **Primary Infertility:** It is a failure to get pregnancy after 1 year of regular intercourse without using contraception.
2) **Secondary Infertility:** It is the incapability to conceive after 1 year of regular intercourse without using contraception following a previous pregnancy or miscarriage.

2.2.2. Causes of Infertility

a. Male Factors (Aditi et al., 2021)

Male infertility may be roughly subdivided into 3 groups:

(1) Hormonal imbalance causing secondary hypogonadism.

(2) Testicular dysfunction (which may be associated with primary hypogonadism).

(3) Obstruction of seminal outflow (usually termed, obstructive azoospermia) and coitus problems.

b. Female Infertility

Can be sub-divided into:

I. Hormonal Disorders

It is an essential reason of anovulation, hormonal disorders that affect ovulation include a hypothalamic\pituitary cause such as hypogonadotropic hypogonadism, or hyperthyroidism, hypothyroidism, and hyperprolactinemia ,women with hormonal disproportion impair producing the folliculogenesis to ensure the development of an oocyte (Olooto *et al.*, 2012).

II. Ovarian Disorders

Disorders of ovulation are often present with irregular periods (oligomenorrhoea) or an absence of periods (amenorrhoea), Studies done worldwide prove that polycystic ovarian syndrome is the single most common cause of female factor of infertility (Deshpande and Gupta, 2019).

III. Fallopian Tube Damage or Blockage

The most common cause is Genital tract infection, which may result in pelvic inflammatory diseases (Gonorrhea and Chlamydia), endometriosis in most cases leads to pelvic adhesions and then tubal obstruction (Reekie *et al.*, 2019).

IV. Uterine Factors

The uterus has a vital role in allowing for a woman to attain pregnancy and carry it to term successfully, there are many etiologies, but by categorizing the diagnoses into either congenital due to structural defect of uterus or acquired due to fibroids (Daolio *et al.*, 2020).

c. Unexplained Infertility

It is defined as 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 *et al.*, 2018).

2.2.3. Evaluation and Management of Infertility

Both men and women partners should submit to simultaneous assessment a reproductive history and examination should include in evaluation. The semen analysis (SA) is a significant element in the initial clinical assessment of the men reproductive health. Semen parameters, which are extremely variable biological measurements and can differ dramatically from ejaculate to ejaculate. The importance of getting at least two SAs, ideally obtained at least one month apart, is highlighted if the first SA shows aberrant values (Schlegel *et al.*, 2021) fig(2-4).

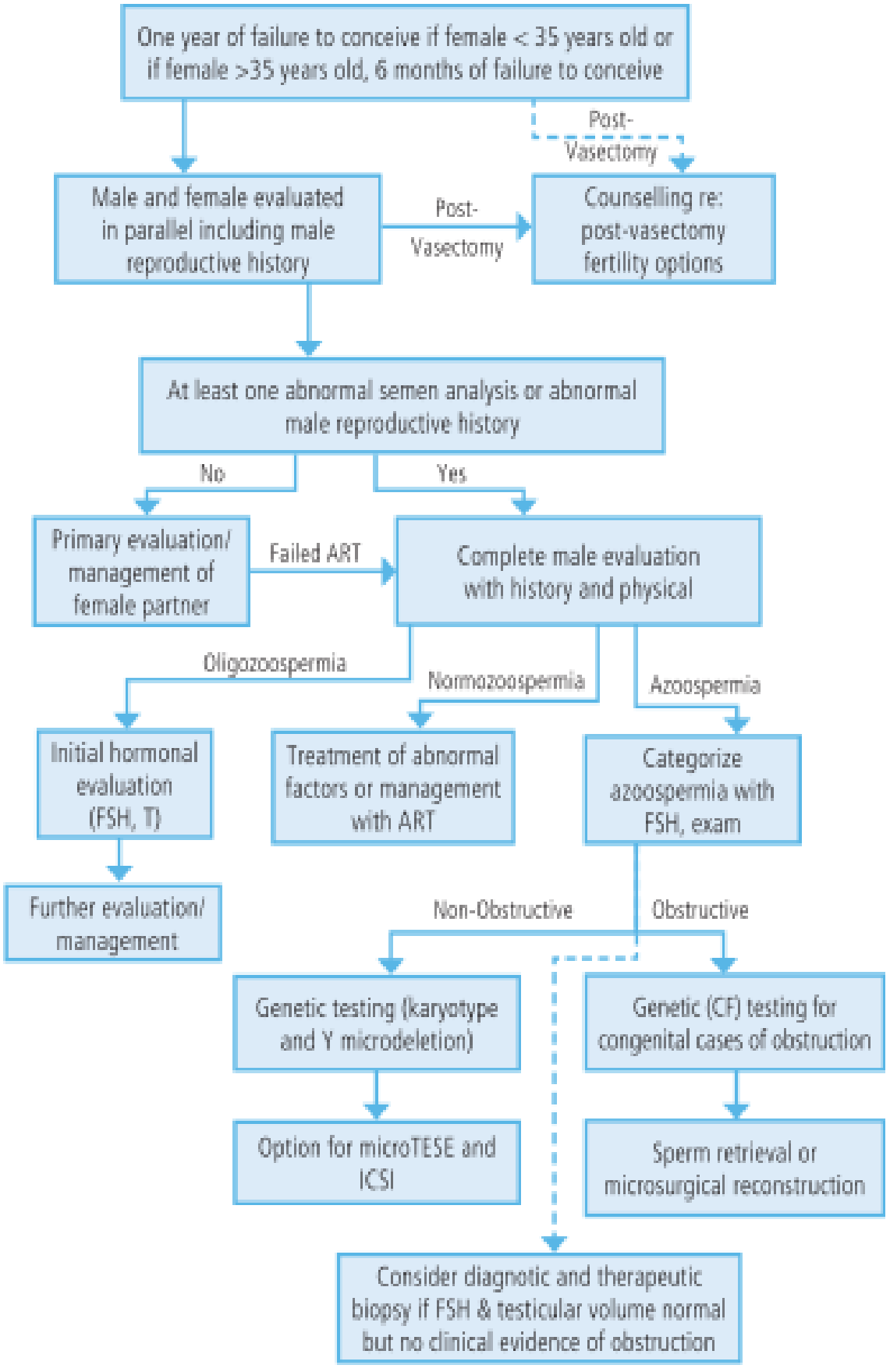

Figure 2-5. Male infertility algorithm (Schlegel *et al.*, 2021).

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

The following are some treatment options for female infertility (Olooto *et al.*, 2012; Bain, 2019):

1) Substances that reduce body weight: In obese, infertile women who are ovulating, a decrease of 5–10% of body weight has been shown to restore reproductive function in 55–100% of women within six months.
2) Human menopausal gonadotrophin is used to induce ovulation (HMG).
3) Bromocriptine in women with hyperprolactinemia.
4) The combination of clomifene citrate and human menopausal gonadotrophin (CC-HMG).
5) Hormone treatment.
6) Surgical assistance.
7) Artificial Insemination (AI): Intrauterine insemination can be used to achieve pregnancy, it is perform in an ovulating lady with patent tubes.
8) In vitro fertilization (IVF): IVF may be used to treat women with endometriosis and damaged fallopian tubes as well as cases of infertility with no known cause.

2.2.4. Factors Affecting Fertility

1) Age

A woman's advancing age is one of the most significant non modifiable risk factors for infertility mainly because of the decrease of gamete's quantity and quality with the passage of the years with its peak between the ages of (18 and 24) years while, it begins to drop after age 27 and then drops at a somewhat larger rate after age 35 years (García *et al.*, 2018).Increased paternal age over 40 years in

some studies or over 50 years in other is associated with decrease in sperm quality, increased DNA fragmentation rates with failure in pregnancy, increased risk of autosomal dominant disorders, impaired neurocognitive development, increased risk of adverse postnatal manifestation of pregnancies with decreased levels of success in IVF, but studies report that when the woman is under 30 years, the increased father's age does not affect fertility rates, fetal quality, and the rate of miscarriages (Masoura *et al.*, 2020).

2) Weight

Nearly one third of the world's population is categorized as being obese (body mass index (BMI) 30 kg/m2) or overweight (BMI 25 to 30 kg/m2). Lower egg quality and quantity, a longer time to conceive, and larger dosages of ovulation-stimulating medicine have all been linked to a higher BMI (Hunter *et al.*, 2021).

Obesity is also related with male infertility, due to hormonal changes secondary to excess adipose tissue, opposite relationship between (BMI) and testosterone, testosterone-to-estradiol ratio, ejaculate volume, sperm concentration, and morphology ,the authors also stated higher rates of azoospermia and oligospermia among obese men related with men of normal weight (Rubin, 2020).

3) 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).

4) Alcohol

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 *et al.*, 2019).

In men shown a significant decline in testosterone levels, seminal fluid volume and sperm concentration in chronic alcoholics (De Angelis *et al.*, 2020).

5) Environmental Factors

Strong evidence showed that exposure to environmental contaminants could interfere with adult female and male reproductive function, these contaminants include heavy metals, organic solvents, pesticides and endocrine disrupting chemicals that have a potential risk factors for infertility and adverse pregnancy outcomes (Ma *et al.*, 2019).

6) Nutrition

Since female reproduction requires a lot more energy than male reproduction does, ovarian activity is repressed in eating disordered women. High-fat diets have an impact on the physical and molecular composition of sperm cells as well as the growing pregnancy and offspring. According to studies, eating meals high in fruits, vegetables, legumes, and seafood consistently is associated with better sperm quality and a lower DNA fragmentation score than people who do not consume such items regularly. Regular consumption of red meat was inversely related to sperm quality (Emokpae and Brown, 2020).

2.3 Assisted Reproductive Technology (ART)

Assisted Reproductive Technology are a group of medical procedures for treating the infertile human in which both male and female gametes are used outside the body (in vitro) to attain pregnancy. It include ;in vitro fertilization (IVF), and intra cytoplasmic sperm injection (ICSI) and other techniques (Mohammadi *et al.*, 2020).

Assisted Reproductive Technique (ART) has been using increasingly over the years as infertility treatment globally between women of different ages since the first baby Louise Brown was born as a result of in vitro fertilization in the UK in 1978 (Lepore and Petruzziello, 2021).

2.3.1 Intra-Cytoplasmic Sperm Injection (ICSI)

It is a type of (ART), and has become the most commonly used technique of in vitro fertilization. It was primarily presented to manage male infertility with severely impaired characteristics such as azoospermia or severely compromised sperm parameters (concentration , motility, and morphology), although the reliability of ICSI has made it an attractive option even in non-male factor couples suffering infertility worldwide (Geng *et al.*, 2020).

2.3.2. Indication of ICSI

A) Male Factor Infertility

Include any factor that inhibit the spermatozoon from normally entering and fertilizing an oocyte due to either (O'Neill *et al.*, 2018; Lacey *et al.*, 2021; Haddad *et al.*, 2021)

1) A low number (Oligozoospermia)of spermatozoa,

2) Impaired motility(Asthenozoospermia)

3) An abnormal morphology(Teratozoospermia)

4) Combination of all(Oligoasthenoteratozoospermia).

5) If there is no sperm (Azoospermia) spermatozoa can be obtained by testicular sperm extraction (TESE or micro-TESE).

6) Reflux of semen into the bladder occurs when the bladder neck fails to close during the expulsion phase (retrograde ejaculation).

7) Electro ejaculation in men with neurologic impairment and present with abnormalities or failure of ejaculation.

8) Immunological infertility presence of anti-sperm antibody in either male or female partner.

B) Non-male Factor Indications (Pereira and Palermo, 2018)

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

6) Unexplained infertility.

2.3.3. Assisted Reproductive Technology (ART) Complications

1. Ovarian Hyper-Stimulation Syndrome (OHSS)

It is an urgent complication that occurs in 3% to 8% of all COH cycles and is more frequently associated with injectable infertility drugs than oral infertility drugs. Due to large quantities of vascular endothelial growth factors generated by the ovaries in response to sharp elevations in hCG, it happens because of increased vascular permeability. A third-spacing of fluid results from the increase in vascular permeability, which also causes ascites, lower extremity edema, intravascular hypovolemia, and reduced renal perfusion (Hilbert and Gunderson, 2019).

1. Ectopic Pregnancy

An ectopic pregnancy is more likely to occur in pregnancies brought on by an ART procedure, with tubal factor infertility as the main risk factor. Recent data show that day five blastocyst transfer reduces the risk of ectopic pregnancy compared to day three, and frozen-thawed cycles reduce the risk of ectopic pregnancy compared to fresh cycles. Therefore, one frozen-thawed blastocyst transfer may be the best option for lowering the incidence of ectopic pregnancy among ART patients (Karadağ and Çalışkan, 2020).

2. Multiple Pregnancy

It is the most common and avoidable treatment-related adverse effect of (ART) due to the transfer of many embryos which is linked with increased fetal/neonatal and maternal risks and a significant request on health resources. The restriction of the number of embryos transferred to the uterus has been gradually applied over the last three decades to decrease this complication (Kadir and Veleva, 2020).

3. Emotions and Psychology

The stress occurs before, during, and after the treatment of ICSI has multiple dimensions. The process is often physically hard and may involve frequent blood tests, ultrasounds, daily hormone injections, and surgery to retrieve oocytes ,waiting to hear about fertilization waiting for a pregnancy result in addition to the cost all are sources of emotional stress (Nicoloro-SantaBarbara *et al.*, 2018).

4. Birth Defect

Singleton infants conceived with ART were 18% more likely than naturally conceived infants to have a major non-chromosomal birth defect; the risk increased to 30% with ICSI in the absence of male factor, with male factor the risk increased to 42% with higher birth defects rates between twins in comparison to singletons (Luke *et al.*, 2021).

2.3.4. Factors Affecting ICSI Outcome

1. Female Related Factors

The age of female partner is the most important part for the success of ICSI. As the chance of pregnancy after the complete ICSI treatment course reduces as the age increases mainly in women >35 years old. From that, we can determine that the age-related decrease in the IVF success rate mostly due to the progressive declined ovarian reserve, with a following lessening in the oocytes quantity and quality (Yang *et al.*, 2018).

Obese women need higher doses of gonadotropins and provide less oocyte. The best BMI for successful ART is between 19 - 30 kg/m2 and is diminished outside this range (Esteves *et al.*, 2018).

2. Type and Duration of Subfertility

Secondary and lower the duration of subfertility associated with better outcome than primary and longer duration of subfertility (Mamsen *et al.*, 2021).

3. The Response to the Stimulation Protocol

If the response of the ovary is Suboptimal (peak E2 level is lower than 300-500pg/ml and/ or the number of formed follicles is beneath 3 -5 on the day of HCG injection) the chance of pregnancy is low after ICSI (Chu *et al.*, 2018).

4. Type of Stimulation Protocol

The long protocol provides better pregnancy rate than the short protocol. There is a 4.6% rise in the clinical pregnancy rate with the use of long protocol. The long control of LH secretion and completion of its suppression is supposed to be the cause behind the better result associated with the long protocol (Bosch *et al.*, 2020).

5. The Quantity and Quality of Transferred Embryos

The success of IVF cycles is mainly dependent on age, quality of the embryo, and endometrial receptivity which is considered as a key factor in the success of IVF. The probability of being pregnant relates with the number and good quality of transferred embryos with correlation between blastocyst embryo transfer and higher success pregnancy rates (Zhang *et al*., 2018).

6. Male Factors

The development of ICSI decreased the influence of male factor, resulting in millions of pregnancies worldwide for couples who without ICSI, would have had little chance of having their own biological child (Palermo *et al.*, 2017).

2.4. Thyroid Hormone

The regulation of cellular differentiation and metabolism by thyroid hormones occurs in essentially all nucleated cells, and abnormalities of thyroid function can appear in a variety of ways (Rosales *et al.*, 2020).

The physiological Effects of Thyroid Hormones are: (Bulur *et al.*, 2020); (Koyyada and Orsu, 2020); (Shahid *et al.*, 2018); (Rosales *et al.*, 2020)

1. Increases the basal metabolic rate.

2. Depending on the metabolic status, it can induce lipolysis or lipid synthesis.

3. Activation the metabolism of carbohydrates.

4. Anabolism of proteins. Thyroid hormones can also induce catabolism of proteins in high doses.

5. Permissive catecholamines impact.

6. Thyroid hormones increase bone growth in children by collaborating with growth hormone.

7. The role played by thyroid hormone in the CNS is significant. It is necessary for the growth of the brain during the prenatal period. It can influence adult mood. Anxiety and irritation can be brought on by hyperthyroidism. Reduced memory, slurred speech, and fatigue are all possible effects of hypothyroidism.

8. Ovulation and menstruation are impacted by thyroid hormone..

Thyroid hormones are vital for good adult health, fetal development, and pregnancy.

Thyrotoxicosis involve symptoms and signs that arise from excess amounts of circulating thyroid hormones. Seventy-five percent of cases are caused by Graves' disease, fifteen percent by multi-nodular goiter, and five percent by toxic adenoma. Less often occurring conditions include TSH-secreting pituitary tumors, factitious temporary thyroiditis (de Quervain's, post-partum), and iodide-induced by drugs or supplements (Novodvorsky and Medicine, 2017).

Primary hypothyroidism affects 1 in 100 people, with a female to male ratio of 6 to 1. More than 90% of cases where the population is not iodine-deficient are caused by autoimmune illness (Hashimoto's thyroiditis) and thyroid failure following I^{131} or surgical therapy of thyrotoxicosis. Secondary hypothyroidism, transitory thyroiditis, amiodarone-induced hypothyroidism, and dyshormonogenesis are less common (Koyyada and Orsu, 2020).

2.4.1. Thyroid Hormone Regulation

The hypothalamus releases thyroid-releasing hormone (TRH), which stimulates thyrotrophs of the anterior pituitary to secrete thyroid-stimulating hormone (TSH). TSH triggers the thyroid follicular cells to release thyroxine T4 (80%), and triiodothyronine T3 (20%), the synthesis of thyroid hormones is dependent on the availability of iodide. T4 must be peripherally converted into T3 in order to contact the thyroid hormone receptor (TR) and become physiologically active. At the cellular level, the interplay of deiodinase enzymes controls this conversion (Pirahanchi *et al.,* 2021).

Deiodinase enzymes are able to remove an iodine atom from a TH isoform's inner or outer ring. T4 outer ring deiodination causes the production of T3, which is the active form of the hormone. T4's inner ring deiodination produces the reverse T3 as a byproduct (rT3), rT3 was once thought to be an inactive TH metabolite since it cannot bind the TR, but recent research has shown that it can have non-genomic impacts like actin remodeling and brain development, deiodinases are differentially expressed between various cell and tissue types (van der Spek *et al.,* 2017).

T4 and T3 can then exert negative feedback on TSH levels ,with high levels of T3/T4 decreasing TSH, and low levels of T3/T4 increasing TSH levels (Benvenga and Mahjub., 2021) as in figure 2-5.

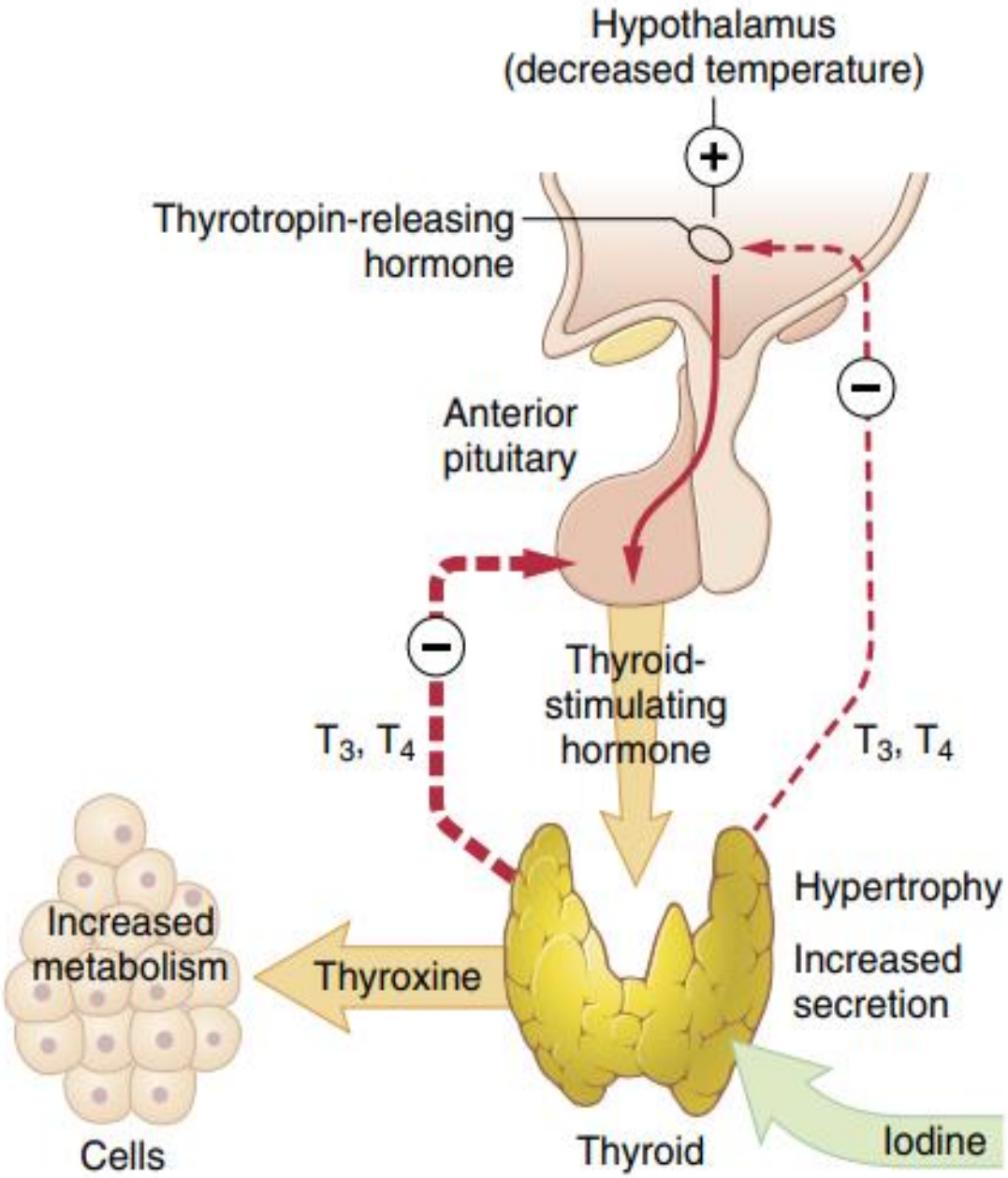

Fig 2-6. Regulation of thyroid secretion. T3, triiodothyronine; T4, thyroxine (Guyton and Hall, 2019).

2.4.2. Thyroid Function Test

Thyroid stimulating hormone (TSH) concentrations are the primary initial test to rule out thyroid illness, taking into account trimester-specific reference values if the patient is pregnant. Immunoassays that assess free T4 (FT4) may be affected by both endogenous and external influences.

Uncertainty surrounds the usefulness of free T3 (FT3) measurement. The existence of antibodies against thyroid peroxidase is a good indicator of thyroid autoimmunity (Visser and Peeters, 2020).

The finding of an abnormal TSH level must be followed by measurements of circulating thyroid hormone levels to confirm the diagnosis of hyperthyroidism (suppressed TSH) or hypothyroidism (elevated TSH). Automated immunoassays are widely available for serum total T4 and total T3 (Borzouei and Mahjub, 2020).

T4 and T3 have a high protein binding capacity, and a variety of circumstances (disease, drugs, genetic factors, etc.) can affect protein binding. In order to determine the amount of biologically accessible hormones, it is useful to evaluate the levels of free, or unbound, hormones. When estrogens (from pregnancy, oral contraceptives, hormone therapy, tamoxifen, selective estrogen receptor modulators, inflammatory liver disease) cause a rise in thyroxin binding globulin (TBG), the total thyroid hormone levels rise as a result, and decreased when TBG binding is reduced as in nephrotic syndrome (Bulur *et al.*, 2020).

2.4.3. The Relation between Thyroid Function and Fertility

One of the most prevalent endocrine issues in reproductive age is thyroid disease, which can lead to infertility as well as menstruation and ovulation disturbances. The exact mechanism by which thyroid diseases affect menstruation and infertility is not precisely understood (Kabodmehri *et al.*, 2021).

Men who have hypothyroidism are more prone to experience libido loss than those who have hyperthyroidism, which can occasionally result in impotence. Lack of thyroid hormone in women frequently results in polymenorrhea and menorrhagia (excessive menstrual bleeding) and (frequent menstrual bleeding) respectively. Other women may experience irregular periods and, on rare occasions, amenorrhea (absence of menstrual blood) due to a deficiency of thyroid hormone (Guyton and Hall, 2019).

Studies mentioned that different cells in the ovary, including oocytes, granulosa cells, and epithelium express receptors of thyroid hormone with THs and its receptors also found in follicular fluid, these receptors may contribute to the regulation of ovarian function, animal researches have showed that thyroid hormones increase follicle stimulating hormone-induced preantral follicular development and interrelate with FSH to suppress granulosa cell apoptosis (Rao *et al.*, 2020).

In addition, they influence the release of (GnRH) in the hypothalamic-pituitary-gonadal axis and interact with a variety of other hormones and growth factors, including estrogen, prolactin (PRL), and insulin-like growth factor (IGF). As a result, subfertility or infertility may result from changes in the levels of thyroid hormones, such as in case of hypo- or hyperthyroidism (Silva *et al.*, 2018).

There is a significant high (PRL) level in infertile women with hypothyroidism when compared to euthyroid patients, untreated hypothyroidism during pregnancy can lead to fetal deaths, premature deliveries, and abortions. So, women wishing for pregnancy and infertile women should be evaluated for thyroid hormones and serum PRL (Koyyada and Orsu, ٢٠٢٠).

Several researches have showed that serum-TSH levels are linked with controlled ovarian hyper stimulation (COH), which is an essential component of ART.COH induces a sharp and supra-physiologic increase of estradiol (4000-6000 ng/L). Estradiol rise induces in turn an increase in thyroxine Binding Globulin (TBG), resulting in the decrease of free thyroid hormone, with a specular TSH elevation. Furthermore, the administration of hCG exerts a thyrotropic effect, that sequentially induces a decrease of TSH levels (Negro, 2018).

Additionally, the period after COH is the "implantation window," which includes the early stages of embryo development. Thyroid hormone plays an essential role in endometrial preparation for pregnancy and initial trophoblast development (Li *et al.*, 2021).

As thyroid receptors are present in addition to the human granulosa cells also found in the endometrium and are distributed differently, the change in TSH levels has been linked to a sub-optimal environment for the implantation and development of the embryo. Therefore, it is crucial for women trying to conceive to maintain optimal TSH levels, particularly in those who are undergoing ART (Jin *et al*., 2019).

In euthyroid state where TSH normal the total effect of COH is considered insignificant while in case of hypothyroidism in which TSH level high the miscarriage rate is higher (Cai *et al.*, 2019).

So Regarding the ART process, the total number of oocytes retrieved may be not affected by thyroid function. Therefore,

levothyroxine is of no benefit to increase the total oocyte retrieval rate. Fertilization rates and embryo quality might be impaired in female with TSH levels >4.0 mIU/L and may be better with levothyroxine therapy (Poppe *et al.*, 2020).

As well as women with hyperthyroidism have significantly reduced chance of a live birth per embryo transfer, related to embryo transfers in female without thyroid disorders, the cause for the reduced chance of a live birth could be linked to inappropriate implantation or early development of embryo in the uterus, although the reasons until now are speculative (Jølving *et al.*, 2019).

The crucial processes of oocyte and follicular maturation occur in a follicular fluid environment, therefore it is critical to thoroughly identify the precise elements involved in this process. Despite the fact that there is a lot of evidence highlighting the thyroid role significance in both natural and assisted reproduction, the thyroid role participation in the ART process has been less studied (Rosales *et al.*, 2020).

Chapter Three

Patients, Materials and Methods

Patients, Materials and Methods

3.1. Patients and Study Design

3.1.1. Study Design

This study was designed as a prospective Cohort study. It had been permitted by the Local Ethical Committee research in college of Medicine/ University of Babylon. Informed consent had been taken from all couples participated in this study.

3.1.2. Research Setting

Fifty sub-fertile couples from the ICSI attenders in the Fertility Center Al-Kafeel super-speciality hospital were involved in the study within the period from July 2021 to July 2022.

The couples had been evaluated by a gynecologist in addition to urologist.

The assessment based on medical history such as the age, date of marriage, cause of infertility, duration, parity, miscarriage if present.

The gynecological, medical, and surgical history of the female partner were reviewed and physical examination including anthropometric measurements (height, weight as well as BMI) and initial investigations for infertility assessment.

Samples of serum and follicular fluid were taken. The ethical committee of the department reviewed and approved the procedures.

3.2. Selection Criteria

3.2.1. Inclusion Criteria

1. Male partners with either normal seminal fluid analysis or mild to moderate impairment of semen parameters based on WHO (2021).
2. Women aged between 19 and 46 years.
3. Unexplained infertility.

4. Semen analysis in which we can get motile and morphologically normal sperms (sever impairment of semen fluid analysis parameters).

3.2.2. Exclusion Criteria

1. Poor responder female (Oocyte number less than 5).
2. Female with frozen embryo transfer.
3. Couples with sperms obtained via testicular sperm extraction.
4. Female with hypo/hyper thyroidism.

Figure (3-1) explain the study design in a summarized way.

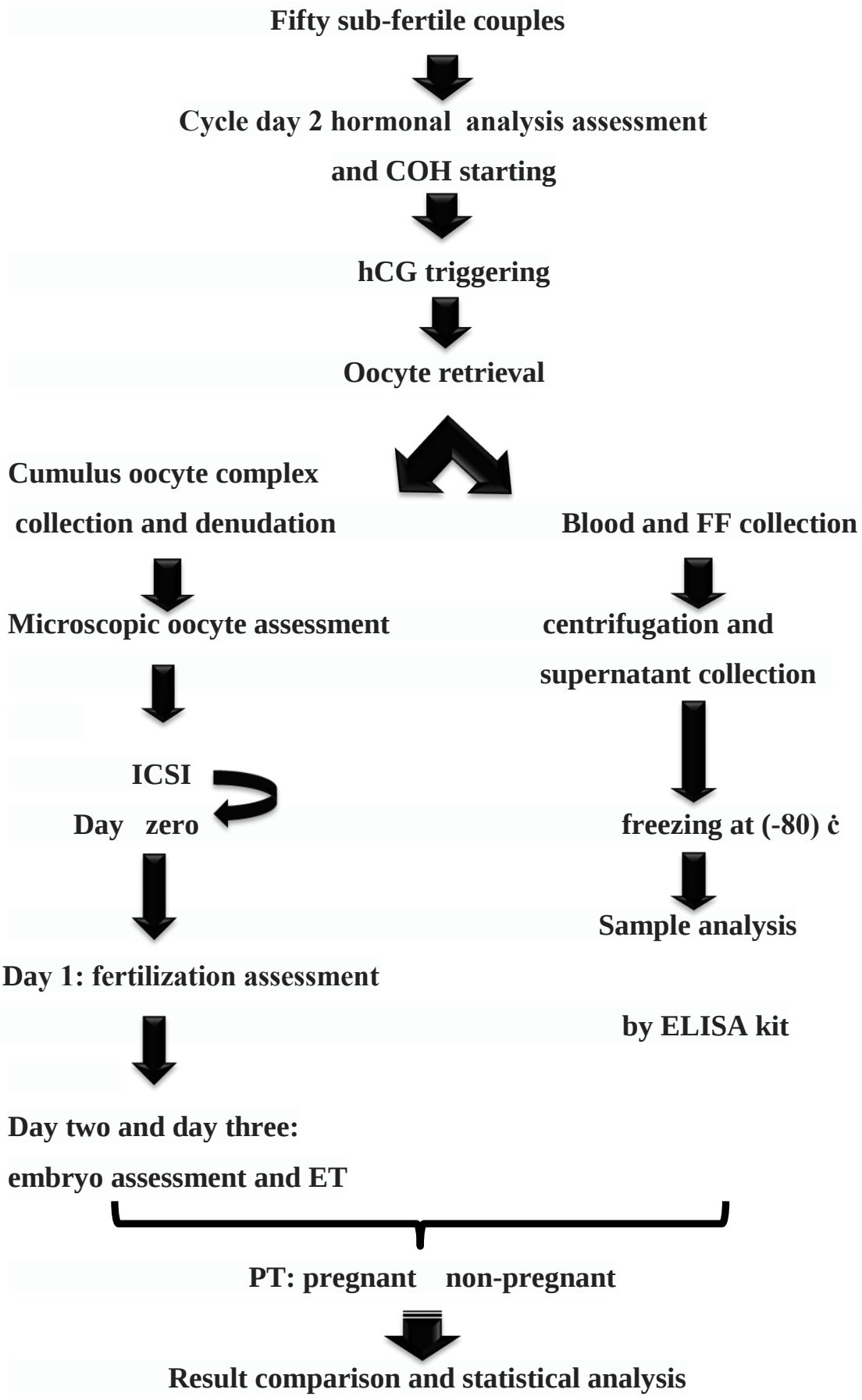

Figure 3-1. Study design

3.3 Materials

3.3.1. Chemicals and culture media

Table 3-1 Main chemical and culture media

Materials	Company , origin
Aspiration media	Fertiucult , Belgium
Hyaluronidase	Fertipro , Belgium
Mineral oil	Ferticult , Belgium
Gain media	Ferticult , Belgium
HEPES media	Ferticult , Belgium
Polyvinylpyrrolidone 10%	Fertipro , Belgium

3.3.2. IVF Lab Devices and Equipment

Table 3-2 In vitro fertilization laboratory devices and tools

Tools and equipment	Company , origin
Air incubator	Heraeus ,kelvitrout ,Germany
Centrifuge	Universal 16 A ,Germany
Co_2 incubator	HERA, USA
Conical tube	Falcon , USA
Culture dish	Nunc , Denmark
Disposal tips	Finntip,thermoscientific , USA
EZ- strip pipette	RI , UK
Four well dish	Nunc , Denmark
Holding pipette for ICSI	Cook , Australia
Injecting needle for ICSI	Cook , Australia
Inverted microscope	Olympus optical Co. Ltd. Japan
Dissecting microscope	Olympus optical Co. Japan
IVF work station	K-system , UK
Weight scale	(Beurer GmbH) Germany

Height scale	(Floor type EVERICH, china)
1 ml syringe	Codan , Denmark
Latex gloves	Comfit. Malaysia

3.3.3. Marker Used

Thyroid hormones level in the blood and FF measured by using enzyme immune-assay ELISA kit for human (snibe, china), that have been confirmed for thyroid hormones level calculation in human blood and FF, as per manufactures' instructions.

A\ Principles of the Test

Sandwich immunoluminometric assay; Use an anti-TSH, anti- T4, anti-T3 monoclonal antibody to label ABEI N-(aminobutyl)-N(ethylisoluminol), and use another monoclonal antibody to label FITC (fluorescein isothyocyanate).

ABEI Label, FITC Label, and magnetic microbeads coated with sheep anti-FITC are thoroughly mixed with the sample, calibrator, or control before being incubated at 37°C to form a sandwich. After sedimenting in a magnetic field, the supernatant is decanted before going through a single cycle of washing.

The starter reagents are then introduced, and a flash chemiluminescent reaction is subsequently started.The light signal is measured by a photomultiplier as RLU (relative luciferase/luminescent units) within 3 seconds and is proportional to the concentration of TSH,T4 or T3 present in samples.

B/ Preparing the Reagent Integral: To avoid foam formation, the Reagent Integral must be horizontally shaken gently and carefully before the sealing is removed.

Remove the sealing, then move the magnetic microbeads compartment's little wheel back and forth until the suspension turns brown. The Integral should be placed in the reagent area and left there for

30 minutes. The magnetic microbeads are automatically stirred and fully resuspended at this point.

3.4 Female Preparation for ICSI

3.4.1 Assessment of Ovarian Reserve

The blood sample obtained on day two or three of the female menstrual cycle by venipuncture into 10 ml plain tubes. Each tube was left standing in the rack for at least 15 minutes on room temperature. After that serum separation by centrifugation was the next phase, the samples were centrifuged for 10 minute at 3000 round /minutes. A single aliquot of serum was used for the measurement of E2, FSH, LH, with mini VIDAS immunoassay technique(Gardner *et al.*, 2018).

3.4.2. Measurement of E2 for Follow up

The serial measurement of E2 level (same process mentioned above) 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 (Malathi *et al.,* 2021).

3.4.3. 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 2-10mm), 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 E2, FSH, LH, and prolactin with mini VIDAS immunoassay technique, then according to the finding on examination, U/S, hormonal analysis and patient's information, they underwent COH (Malathi *et al.,* 2021).

Controlled ovarian hyper stimulation (COH) comprises 3 basic elements: (Gallos *et al*., 2017)

- 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 ovulation.
- Triggering of final oocyte maturation (36 to 38) hours prior to oocyte retrieval.

Controlled Ovarian Hyper Stimulation: It is done by either:

• 'long protocol', which is the standard, the GnRH agonist (decapeptyl) is started at least two weeks before stimulation (in the mid-luteal phase of the preceding cycle) and continued up until oocyte maturation is reached (Xu *et al*., 2020).

• 'short protocol' in which the GnRH agonist (decapeptyl) is started at the same time with stimulation (day 2) and persistent up till the day of oocyte maturation reached (Ou *et al*., 2015).

• GnRH antagonists (cetrotide) involve a shorter duration of use, absence of vasomotor symptoms, lower risks of ovarian hyper-stimulation, 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) 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 ovulation (Salama *et al*., 2021).

3.5. Oocyte Pick Up

Which done by gynecologist occur in 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 (Li *et al.*, 2021).

Follicular fluid collection by Pre-warmed tubes and these tubes were then transferred to IVF lab for oocyte searching and collection by embryologist, by using a Petri dish and 1 ml syringe with adapter (Codan) for searching of cumulus oocyte complex (COC) under dissecting stereomicroscope then the COC transferred to a four-well dish containing Gain medium, which was prepared overnight and incubated at 37ċ with 5% CO_2 till time of oocyte denudation (D'Angelo *et al.*, 2019).

3.6. Assisted Reproduction Technologies

3.6.1. Dishes Preparation

1. Preparation of Denudation Dish

A drop of about 100 µl of hyaluronidase solution with 5 drops of HEPES 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 135 and 155 µm (Naji *et al.*,2018).

2. Preparation of Injection Dish

Nine droplets, each one is 5 µL of HEPES buffered medium, arranged in a 3x3 square in a shallow falcon dish and covered with oil. A s well as 5 µL of 10% PVP where the sperms 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 (Naji *et al.*,2018).

3. Culture Dish

A special IVF media with equilibrated mineral oil were incubated in culture dishes overnight in incubator conditions of 5% Co_2 and 37ºC. The culture system for embryo includes micro droplets under oil: 5 drops of culture media of 50-µl volume were arranged with one central drop surrounded by four drops covered by 5 ml of mineral oil then placed in the incubator (Gardner *et al.*, 2018).

3.6.2. Measurement of Serum and Follicular Thyroid Hormone on the Day of Oocytes Retrieval

- **Sample Collection**

Serum and follicular fluid samples were collected at the day of oocytes pick up.

A. Follicular Fluid (FF)

A. Fifty samples, of transparent, follicular fluid were obtained from ovarian follicles at the time of oocyte pick up, with ultrasound guided aspiration needle and emptied into planed tube. The pooled sample from each patient was centrifuged immediately to get out of the debris and the supernatant stored at -80 ºC (D'Angelo *et al.*, 2019).

B. Serum Sample

A blood sample was withdrawn at the day of oocytes pick up by venipuncture and emptied into jelly-tube, left standing in the tube rack for at least 15 minute and then centrifuged for 10 minutes 3000 cycle and serum stored at -80 ºC. The levels of thyroid hormone in the serum and follicular fluid were determined quantitatively using commercial ELISA kits (Gardner *et al.*, 2018).

3.6.3. Oocyte Denudation

Oocytes are enclosed by the cumulus cells forming COC these COC may block the injecter needle and so interfere with the gentle oocyte micro-injection. 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 *et al.*, 2018).

Denudation done by two steps: (Carvalho *et al.*, 2020)

B. Enzymatic Denudation: Because of one of the main component of COC is hyaluronic acid so hyalurodinase 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.

C. Mechanical Denudation: Further denudation was done mechanically by gentle pipetting through capillaries of successively narrower inner diameters of 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.

3.6.4. Assessment of Denuded Oocyte

Under an inverted microscope the oocytes morphologic features were classified according to maturity if MII (mature with polar body), MI (immature no polar body) or germinal vesicle GV (immature with oocyte nucleus), and the existence of intra- and extra- cytoplasmic abnormalities (Faramarzi *et al.*, 2017) fig. (3-2).

GV MI MII

Figure (3-2): Oocyte maturity(GV, MI and MII oocyte)

3.6.5. Sperm Preparation (McVay and Cooke, 2021)

The most method used for the sperms 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 in the incubator at 37ºC for liquefaction completely for about 30-50 minutes.
- 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 loosened with HEPES media.
- The conical tube was incubated at oblique position to maximize the surface area for 30-45 minutes.
- After the incubation time, the upper half was aspirated then mixed and the concentration of the sperm was determined.

3.6.6. Set up of the ICSI Micromanipulator

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

3.6.7. Sperm Immobilization

The sperm of accepted morphology and motility was selected to be gently 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 gently to compress the sperm tail. 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.

3.6.8. The Injection of Oocyte

It was done by using the holding pipette, the oocyte has been held in place by pipette suction force and so the polar body was placed at 6 o'clock so that reduce the damage to the meiotic spindle. The injector, which already contained the immobilized sperm near its tip, was moved close the held oocyte and interjected to the ooplasm of the MII egg at a place of 3 o'clock. Applying the minimal suction was necessary to allow the ooplasm entering the injection pipette. The suction was immediately stopped and the sperm was released to the oocyte gently with head entering first, now the pipette can be carefully withdrawn figure (3-3).

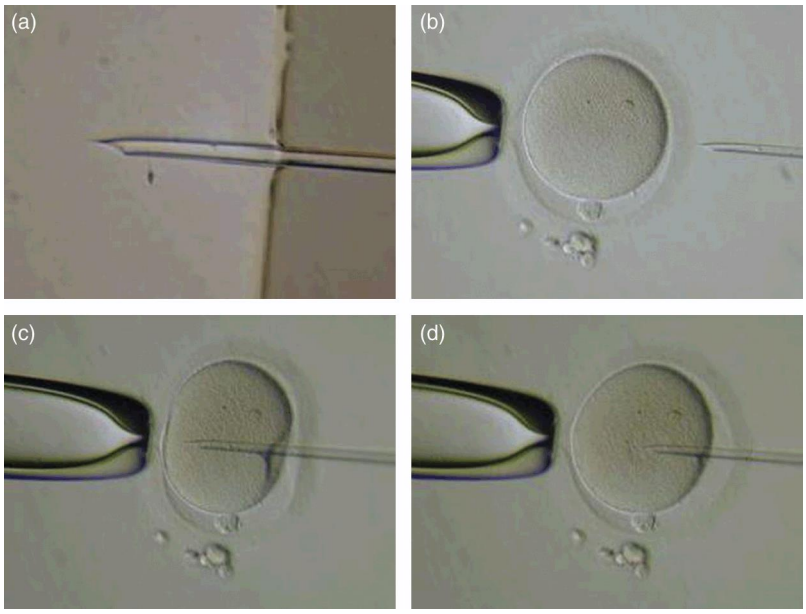

Figure 3-3. sperm immobilization(a) and intra-cytoplasmic sperm injection (b, c and d)

3.6.9. Incubation of the Injected Oocyte and Assessment of Fertilization

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% Co_2 and 37°C. 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 pro-nuclei, which have the nucleoli.

No fertilization **Fertilization**

Figure 3-4. fertilization assessment

3.6.10. Assessment of Embryo Quality

After assessing the oocytes for fertilization, the morphological evaluation of embryo occurred 24-48 hour subsequent to oocyte pick up depending on: cleavage, symmetry of blastomeres, and presence and percentage of fragmentation. Next to ICSI, 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 stage 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. (Table 3-3).

From: Standardization of grading embryo morphology

Growth phase	Overall grade	Stage
Cleavage	Good, Fair, Poor	Cell #: 1 through >8
		Fragmentation: 0%, <10%, 11–25%, >25%
		Symmetry: Perfect, Moderately Asymmetric, Severely Asymmetric

Table 3-3 embryo grading (Racowsky *et al.*, 2010)

3.6.11. 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 'maximally 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 sited in the uterus. This was followed by pulling out the whole catheter and checking it for retained embryos.

3.6.12. luteal Phase Support and Pregnancy Test

Following oocyte pick up, the patients were kept on medication to support the luteal phase. This medication included progesterone vaginal suppositories (200 mg two time per day), Duphastone tablets (10 mg orally two time each day). Two weeks after the embryo transfer pregnancy was checked by evaluating the serum level of β-hCG, a positive level confirmed the pregnancy. The clinical pregnancy was diagnosed by vaginal ultrasound 5- 7 days after the diagnosis of biochemical pregnancy.

3.7. Statistical Analysis

The analysis of the study data done by using the software program: SPSS 26(Statistical Package for Social Sciences). Numerical data were characterized by mean ± standard deviation, whereas the categorical data characterized by numbers and percentages. Independent t-test was used to study the difference between pregnant and non-pregnant women (two groups). The Chi-Square test was used to study the association between categorical data. Pearson correlation was used for detection of correlation between different factors. The Binomial logistic regression analysis for pregnant women against not pregnant women. ROC curve for pregnant women against not pregnant women in studied group was done to detect sensitivity and specificity of thyroid hormones .A two-tailed P value less than 0.05 was considered statistical significant (Daniel,1999).

Chapter Four

The Results

The Results

4.1. The Demographic and Clinical Data of the Studied Patients

Table 4-1 shows the demographic and some clinical data of 50 women underwent ICSI/Embryo transfer participated in this study regarding to the age, body mass index (BMI) , duration, type, and causes of subfertility of the studied Patients: The data are expressed in mean ± SD, minimum-maximum and percentage.

Most studied group had primary infertility 74% and 38% with male factor infertility.

Table 4-1 The demographic and clinical data of the studied patients

Variables		**Values**
Number		50 couples
Female's age(years)		31.52 ± 5.91 (19-46)
Female's BMI (kg/m^2)		27.71± 4.81 (21.6-44.9)
Duration of subfertility (years)		8.53±5.18 (1.5-19)
Type of subfertility	Primary	37 (74%)
	Secondary	13 (26%)
Etiology of subfertility	male	19 (38%)
	female	6 (12%)
	combined	16 (32%)
	unexplained	9 (18%)

BMI; body mass index.

4.2. Biochemical Analysis

4.2.1. Reproductive Hormonal Assay of Studied Patients

Table 4-2 Represent reproductive hormonal assay of studied patients at second day of menstrual cycle (basal E2, AMH, basal FSH,LH and prolactin) and E2 at day of hCG injection, the data are expressed in mean ± SD.

Table 4-2 Reproductive hormonal assay of studied patients

Hormonal assay	Mean ± SD	Reference range
Basal E2 (pg/ml)	41.20±2.44	30-400(pg/ml)
E2 at day of hCG (pg/ml)	2044.10±781.77	-------
AMH(ng/ml)	3.073±0.31	1-3(ng/ml)
Basal FSH(mlu/ml)	5.66±0.27	1.5-12(mlu/ml)
LH(mlu/ml)	4.00±0.38	1.5-15(mlu/ml)
Basal PRL (ng/ml)	19.71±1.90	>25(ng/ml)

E2; estradiol 2, AMH; anti-mullerian hormone, FSH; follicle stimulating hormone, LH; luteinizing hormone, E2; estradiol, PRL; prolactin.

4.2.2. Thyroid Function Test of Studied Patients

Thyroid function test (TSH, T4, and T3) was measured in both serum and follicular fluid and expressed by mean ± SD was shown in table 4-3.

Table 4-3 Thyroid function test of studied patients in serum and follicular fluid

Hormones	Serum mean ± SD	Follicular fluid mean ± SD	Reference range
TSH(µlU/ml)	1.71±0.79	2.28±1.23	0.5-5(µlU/ml)
Total T3(ng/ml)	1.15±0.29	1.53±0.15	0.9-2.33(ng/ml)
Total T4(ng/dl)	7.73±1.77	9.14±1.53	6-12(ng/dl)

S; serum, FF; follicular fluid, TSH; thyroid stimulating hormone, T3; tri-iodothyronin, T4;thyroxine.

4.3. ICSI Outcomes and Pregnancy Rate of Studied Patients

The ICSI outcome was shown in table 4-4 in form of median and Minimum-maximum level.

Table 4-4 ICSI parameters expressed by median (minimum – maximum)

ICSI parameters	Median	(Minimum-maximum)
Oocyte number	12	(5-37)
MII	9	(2-36)
MI	2	(0-5)
GV	1	(0-11)
Injected MII	9	(2-36)
2PN	8	(1-30)
0PN	1	(0-6)
3 or more PN	0	(0-1)
Good (grade A) embryo	3	(0-12)
Fair (grade B) embryo	4	(0-20)
Bad (grade C) embryo	1	(0-10)
Fertilization rate	77.1 %	
Cleavage rate	100 %	

MII(metaphase II), MI(metaphase I), GV(germinal vesicles), PN(pro-nuclei).

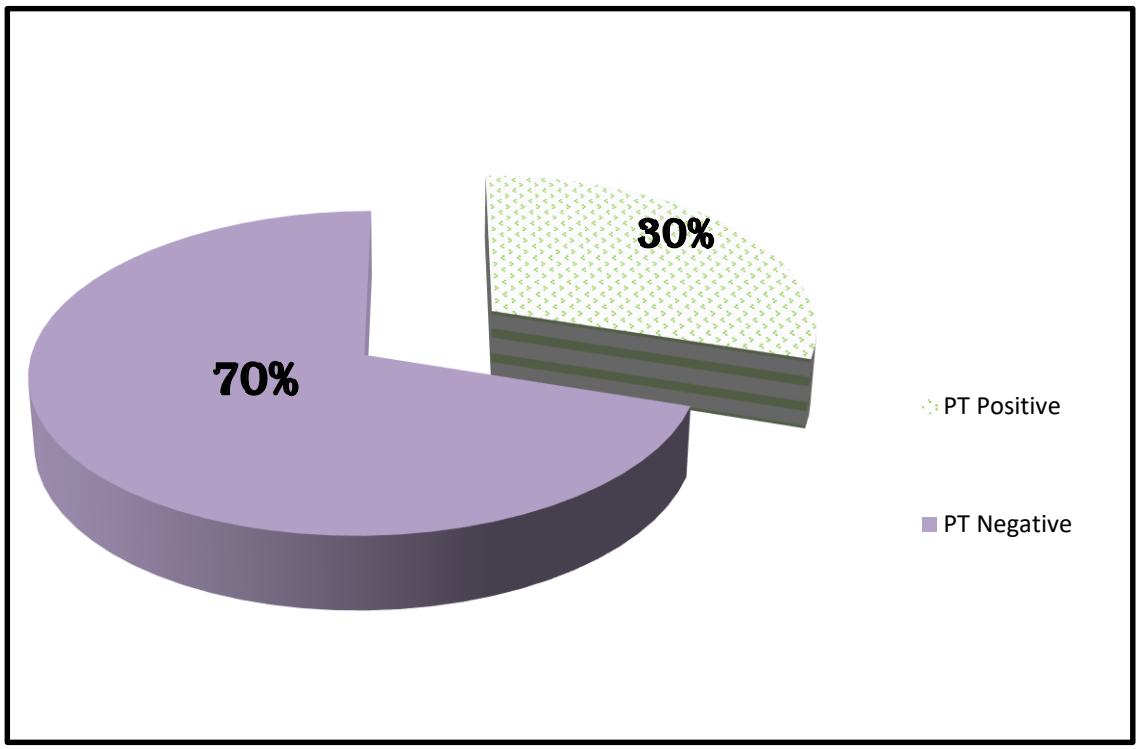

Figure 4-1. The pregnancy rate in the studied group

The pregnancy rate after ICSI was shown in figure 4-1, 30% of the patients in the study group had positive pregnancy test while 70 % had negative result.

According to the occurrence of pregnancy, the studied groupes was divided into: group I: female partner with positive pregnancy test (n=15) and group II: female partner with negative pregnancy test (n=35).

4.3.1. Demographic Clinical Data Differences between Pregnant and non-pregnant Women

The demographic and clinical features (age, BMI, infertility period, type and cause of subfertility) in both groups was shown in table 4-5.There were no statistical significant differences regarding these variables between pregnant and non-pregnant groups (P>0.05).

Table 4-5 The demographic and some clinical features (age, BMI, infertility period, type and cause of subfertility) of the pregnant and non-pregnant groups

Variables		Pregnant (n=15) Mean ± SD	Non-pregnant (n=35) Mean ± SD	p-value
Women's Age (years)		30.86±5.57	31.80±6.11	0.61
Women's BMI (kg/m^2)		26.50±3.43	28.23±5.25	0.24
Duration of subfertility (years)		9.06±5.59	8.30±5.06	0.63
Type of subfertility	Primary	9(60%)	28(80%)	0.13
	Secondary	6(40%)	7(20%)	
Etiology of subfertility	Male	5(33.3%)	14(40%)	0.69
	female	3(20.0%)	3(8.6%)	
	combined	5(33.3%)	11(31.4%)	
	unexplained	2(13.3%)	7(20%)	

P value <0.05 was significant

4.3.2 Reproductive Hormones in Pregnant and non-pregnant Women

The hormonal assay of the pregnant and non-pregnant groups at second day of menstrual cycle (basal E2, AMH, basal FSH, LH and prolactin) and E2 at day of hCG injection was shown in table 4-6. There was statistical significant differences in FSH level between pregnant and non-pregnant groups (P<0.05). While there were insignificant difference regarding E2 , AMH, LH and prolactin levels (P>0.05).

Table 4-6 The hormonal analysis in pregnant and non-pregnant groups (baseline E2, E2 at day of hCG injection, AMH, FSH, LH and PRL)

variable	Pregnant (mean ± SD)	Non-pregnant (mean ± SD)	p-value
E2 baseline(pg/mL)	42.22±19.34	38.81±11.21	0.52
E2 at day of hCG injection (pg/ml)	2114.06±687.93	2014.11±826.35	0.68
AMH (ng/ml)	3.72±3.049	2.79±1.75	0.18
FSH(mlu/ml)	6.73±1.85	5.20±1.80	**0.009***
LH(mlu/ml)	3.58±1.53	4.18±3.12	0.48
PRL(ng/ml)	20.21±14.48	19.49±13.22	0.86

P value <0.05 was significant. E2;estradiol 2, AMH; anti-mullerian hormone, FSH; follicle stimulating hormone, LH; luteinizing hormone, E2; estradiol, PRL; prolactin.

4.3.3. Serum and Follicular Fluid Thyroid Hormone Level Difference between Pregnant and non-Pregnant women

The thyroid function test of pregnant and non-pregnant women was shown in table 4-7. There was statistical significant differences in serum TSH level between pregnant and non-pregnant groups (P<0.05). While there were insignificant difference regarding to serum T4 , serum T3 , follicular TSH , follicular T4 and follicular T3 (P>0.05).

Table 4-7 Thyroid function test (TSH,T4 and T3) in both serum and follicular fluid in pregnant and non-pregnant groups

Thyroid hormones	Pregnant (mean ± SD)	Non-pregnant (mean ± SD)	p-value
S.TSH(µlU/ml)	2.13 ± 0.93	1.53 ± 0.67	**0.01***
S.T4(ng/dl)	6.58 ± 2.91	7.68 ± 1.87	0.75
S.T3(ng/ml)	1.16 ± 0.26	1.14 ± 0.31	0.83
FF.TSH(µlU/ml)	2.27 ± 0.91	2.28 ± 1.35	0.98
FF.T4(ng/dl)	9.14± 1.53	9.20 ± 1.46	0.69
FF.T3(ng/ml)	1.53 ± 0.13	1.52 ± 0.16	0.92

P value <0.05 was significant . S; serum, FF; follicular fluid, TSH; thyroid stimulating hormone, T3; tri-iodothyronin, T4;thyroxine.

4.3.4. Comparison between Pregnant and non-pregnant Groups Regarding the ICSI Outcome

The ICSI outcome in pregnant and non-pregnant women was shown in Table 4-8. which include the number and maturity status (GV, MI and MII) stages of the retrieved oocytes and embryo grading in both groups. It shows a significant statistical difference between pregnant and non-pregnant groups regarding the grade C embryo ($p<0.05$).

Table 4-8 The ICSI outcome in pregnant and non-pregnant women

ICSI parameters	Pregnant (n=15) Median	Non-pregnant (n=35) Median	p-value
Oocyte	14	11	0.08
MII	10	8	0.10
MI	2	2	0.56
GV	1	0	0.68
Injected MII	10	8	0.11
2PN	9	8	0.11
0PN	1	1	0.77
3 or more PN	0	0	0.53
Grade A embryo	3	3	0.79
Grade B embryo	4	3	0.22
Grade C embryo	2	0	**0.02***

P value <0.05 was significant . MII (metaphase II), MI (metaphase I), GV (germinal vesicles), PN (pro-nuclei).

4.4. The Correlation of Serum (TSH, T4 and T3) and Follicular (TSH,T4 and T3)

The correlation between serum thyroid function test (TFT) and follicular TFT was shown in table 4-9. There was a significant positive correlation between serum and follicular TSH (r=0.33 , P=0.01). Also there was significant positive correlation between serum T3 and follicular TSH (r=0.33 , P=0.01).

Table 4-9 The correlation of serum (TSH,T4,T3) and follicular (TSH,T4,T3)

Thyroid function test		**FF.TSH (µlU/ml)**	**FF.T4 (ng/dl)**	**FF.T3 (ng/ml)**
S.TSH(µlU/ml)	r	**0.33***	-0.15	-0.16
	p	0.01	0.27	0.25
S.T3(ng/ml)	r	**0.33***	0.14	0.10
	p	0.01	0.30	0.48
S.T4(ng/dl)	r	0.19	0.12	0.14
	p	0.1	0.39	0.30

P value <0.05 was significant . S; serum, FF; follicular fluid, TSH; thyroid stimulating hormone, T3; tri-iodothyronin, T4;thyroxine

4.4.1.The Correlation of Thyroid Hormones Level with Reproductive Hormones

The correlation between thyroid function test and measured reproductive hormones was shown in table 4-10. There was a significant positive correlation between S.T4 and FSH (r=0.32 , P=0.02) . As well as there was a significant positive correlation between FF.T4 with AMH and FSH (r=0.31 , P=0.024 , r=0.35 , P=0.01).Also there was a significant positive correlation between FF.T3 and E2 (r=0.31 , P=0.02).

Table 4-10 The correlation of thyroid hormones and (Basal E2, E2 at day of hCG injection, AMH, FSH, LH and PRL) hormones

Thyroid hormones		Basal E2	E2 hCG day	AMH	FSH	LH	PRL
S.TSH	r	-0.20	-0.05	-0.01	-0.06	-0.18	-0.12
	p	0.15	0.97	0.90	0.66	0.20	0.38
S.T4	r	-0.24	-0.09	0.26	**0.32****	0.14	-0.09
	p	0.08	0.50	0.06	0.02	0.30	0.50
S.T3	r	-0.03	-0.18	-0.09	0.15	0.11	-0.02
	p	0.83	0.20	0.53	0.28	0.42	0.85
FF.TSH	r	-0.08	0.17	0.05	-0.07	-0.08	-0.19
	p	0.57	0.23	0.70	0.60	0.56	0.18
FF.T4	r	0.16	0.15	**0.31***	**0.35***	0.18	-0.14
	p	0.26	0.27	0.02	0.01	0.19	0.30
FF.T3	r	-0.16	**0.31***	0.26	0.17	0.24	-0.05
	p	0.25	0.02	0.05	0.22	0.08	0.70

P value <0.05 was significant . S; serum, FF; follicular fluid, TSH; thyroid stimulating hormone, T3; tri-iodothyronin, T4;thyroxine

4.4.2. The Correlation of Thyroid Hormones Level with ICSI Outcome

The correlation between thyroid hormones (TSH,T3 and T4) in both serum and follicular fluid and ICSI parameters was shown in table 4-11. S.TSH correlate positively with GV oocyte (r=0.40, P-value =0.00), also S.T3 correlate positively with 3 or more PN in a statistically significant manner r=0.35, P-value =0.01. FF.T4 correlate positively with MII and 2-PN in a statistically significant manner (r=0.28 ,P-value=0.04 ; r=0.30 , p value = 0.03 respectively). FF.T3 significantly positively correlate with oocyte number, MII and 2PN stage (r=0.34, P-value=0.01 ; r=0.35, P-value=0.01; r=0.40, p value = 0.01 respectively).

Table 4-11 The correlation of thyroid hormones (TSH,T4 and T3) in both serum and follicular fluid and ICSI parameters

Thyroid hormones		Oocyte	MII	MI	GV	2PN	0 PN	3 or more PN
S.TSH	r	0.05	-0.08	0.13	**0.40****	-0.02	0.03	-0.07
	p	0.68	0.54	0.36	0.00	0.87	0.79	0.60
S.T4	r	0.10	0.08	0.24	0.05	0.02	-0.10	-0.03
	p	0.45	0.56	0.08	0.73	0.85	0.45	0.78
S.T3	r	0.10	0.09	0.20	0.05	0.07	0.15	**0.35***
	p	0.46	0.35	0.15	0.70	0.59	0.29	0.01
FF.TSH	r	0.17	0.09	-0.03	0.19	0.17	0.05	0.21
	p	0.22	0.52	0.79	0.18	0.23	0.72	0.14
FF.T4	r	0.25	**0.28***	0.25	-0.05	**0.30***	0.02	-0.12
	p	0.07	0.04	0.07	0.69	0.03	0.86	0.40
FF.T3	r	**0.34****	**0.35***	-0.16	-0.16	**0.40****	-0.10	-0.04
	p	0.01	*0.01	0.24	0.25	0.01	0.46	0.78

P value <0.05 was significant: S; serum, FF; follicular fluid, TSH; thyroid stimulating hormone, T3; tri-iodothyronin, T4;thyroxine

The correlation between thyroid hormones (TSH, T3 and T4) in both serum and follicular fluid and embryo quality was shown in table 4-12.

FF.T4 positively correlate with fair quality embryos in a statistically significant manner (r= 0.35 ,P-value=0.01). FF.T3 correlate positively with good and fair quality embryos in a statistically significant manner (r=0.32, P-value=0.02 ; r=0.33, p value= 0.01 respectively).

Table 4-12 The correlation of thyroid hormones (TSH, T4 and T3) and quality of embryos

Thyroid hormone		**Good**	**Fair**	**Bad**
S.TSH	r	-0.11	-0.02	0.13
	p	0.43	0.88	0.34
S.T3	r	-0.18	-0.14	0.17
	p	0.18	0.32	0.22
S.T4	r	-0.06	0.03	0.05
	p	0.67	0.83	0.72
FF.TSH	r	0.06	0.18	0.03
	p	0.65	0.20	0.83
FF.T4	r	0.01	**0.35***	0.19
	p	0.90	0.01	0.18
FF.T3	r	**0.32***	**0.33****	0.08
	p	0.02	0.01	0.56

P value <0.05 was significant Abbreviation: S; serum, FF; follicular fluid, TSH; thyroid stimulating hormone, T3; tri-iodothyronin, T4;thyroxine

4.5. Binary Logistic regression for thyroid function test in pregnant women against non-pregnant women

Binary Logistic regression analysis for thyroid function test in pregnant women against non-pregnant women was shown in table 4-13.

Odds ratio for S.TSH equal to 2.64 [1.13-6.17]. Increase S.TSH within specific limit associated with success pregnancy about three times when compared with failed group .

Table 4-13 Binary Logistic regression analysis for thyroid function test in pregnant women against non-pregnant women

TFT	P-value	Odds ratio	95% Confidence Interval	
			Lower limit	Upper limit
S.TSH	**0.001**	2.64	1.13	6.17
S.T4	0.75	1.05	0.75	1.48
S.T3	0.83	1.25	0.16	9.75
FF.TSH	0.98	0.99	0.60	1.63
FF.T4	0.68	0.92	0.61	1.37
FF.T3	0.91	1.22	0.02	62.60

P value <0.05 was significant S; serum, FF; follicular fluid, TSH; thyroid stimulating hormone, T3; tri-iodothyronin, T4;thyroxine.

a. The reference category is: non pregnant group.

4.6. ROC Curve of Serum and Follicular Thyroid Function Test for Pregnant in Contrast to non-pregnant women

Figure 4-2: ROC curve for serum and follicular thyroid hormones(serum TSH, serum T3, serum T4, follicular TSH, follicular T4, and follicular T3) in pregnant against non-pregnant women.

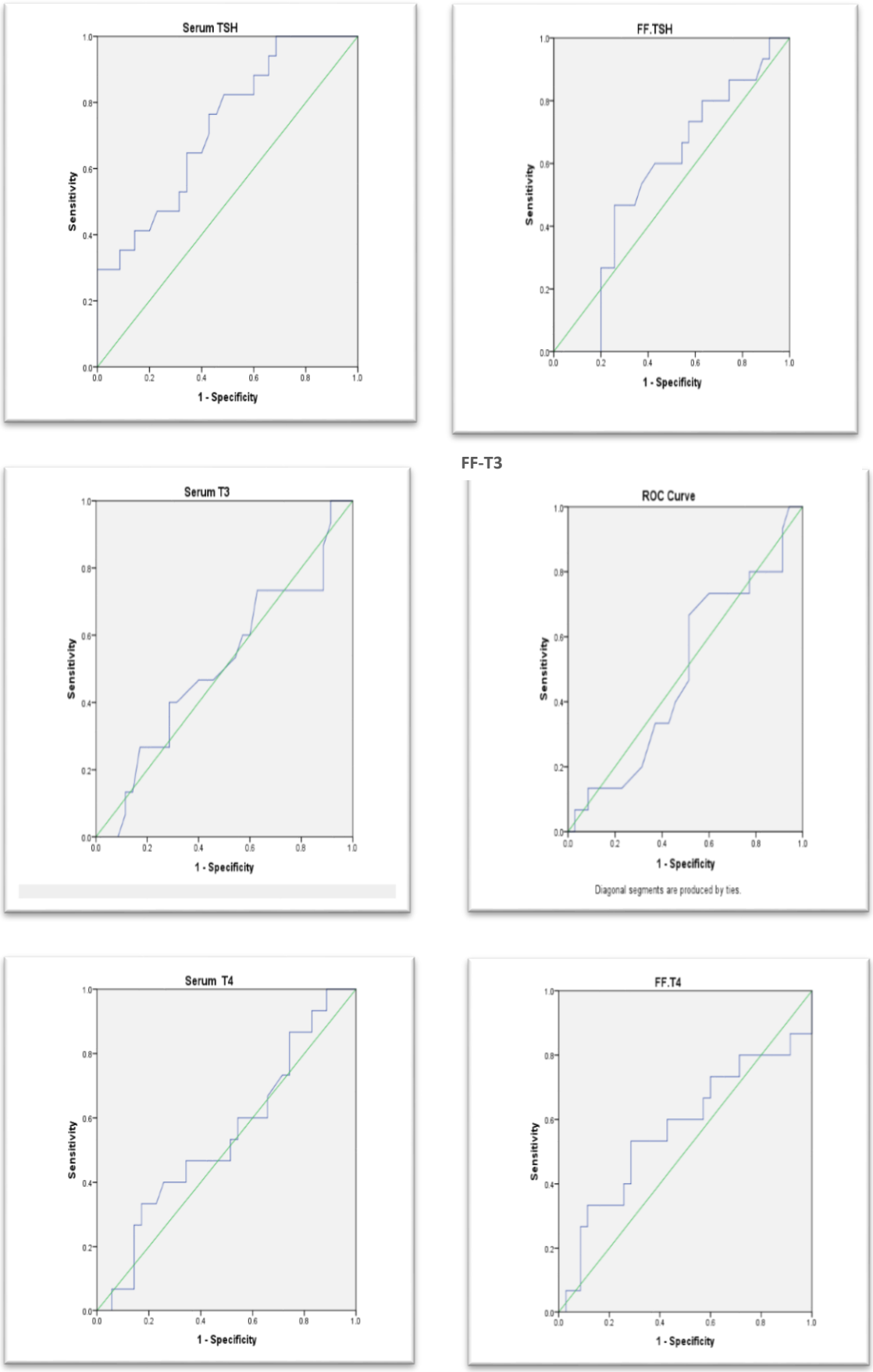

Figure 4-2. Receiver operating characteristic curve

Table 4-14 Receiver operating characteristic curve in pregnant against non-pregnant women

TFT	AUC	Cut of value	Sensitivity	Specificity	95% Confidence Interval	
					Lower Bound	Upper Bound
S.TSH	0.70	1.50	80%	72%	0.53	0.83
FF.TSH	0.56	2.00	60%	58%	0.49	0.72
S.T3	0.54	1.00	51%	63%	0.33	0.69
FF.T3	0.51	1.50	56%	55%	0.31	0.62
S.T4	0.55	9.00	60%	50%	0.37	0.71
FF.T4	0.60	9.00	66%	63%	0.38	0.75

S; serum, FF; follicular fluid, TSH; thyroid stimulating hormone, T3; tri-iodothyronin, T4;thyroxine.

Receiver operating characteristic curve (ROC) curve was done , serum TSH had higher area under the curve , cut of value of 1.5 give a sensitivity 80% and specificity 72% followed by FF.T4, cut of value of 9 give a sensitivity 66% and specificity 63%. So serum TSH could be considered as a good predictor of success ICSI as shown in figure 4-2. While other thyroid hormones tests of both serum and follicular fluid show insignificant association to pregnancy outcomes.

Chapter Five

Discussion

Discussion

5.1. The Demographic and Clinical Data of the Studied Patients

Fifty sub-fertile couples involved in the study with a mean age (31.52±5.91), mean BMI (27.71±4.81) and a mean of sub-fertility duration (8.53±5.18).

There was 74% of couples complain of primary infertility in contrast of 26% of them complain of secondary infertility.

According to the etiology of sub-fertility the majority of the study group was male factor (38%) and thus (62%) distributed between female, combined and unexplained infertility.

5.2. Biochemical Analysis

5.2.1. Reproductive Hormonal Assay of Studied Patients

Regarding to the reproductive hormones there were in the normal reference range.

5.2.2. Thyroid Function Test of Studied Patients

According to the thyroid function test in serum and follicular fluid, the hormones levels were also within the normal reference range.

5.3. ICSI Outcomes and Pregnancy Rate of Studied Patients

The ICSI parameters of the study sample result in a median oocyte number equal to 12 with MII oocytes median equal to 9 and 2 PN fertilization equal to 8 thus the fertilization rate equal to 77.1% and cleavage rate equal to 100%.

The pregnancy rate in the present study was 30% and this percentage was comparable with that stated in (ESHRE, 2019) which was 24.3% reflecting the high quality of Iraqi centers to achieve a high results.

5.3.1. Demographic Clinical Data Differences between Pregnant and non-Pregnant Women

Regarding the age , the age of pregnant women insignificantly lower than non-pregnant women (P>0.05). Hassan and Zahir, 2020, and Chen *et al.*, 2022 studies mentioned that the female age was the main predictor of pregnancy but its significant effect in decreasing pregnancy rate appear if age was increased more than 35 years.

About BMI , the BMI of pregnant women insignificantly lower than non-pregnant women (P>0.05) and that agreed with many studies including Shah *et al.*, 2011 which mentioned that no such an effect of BMI on pregnancy rate unless BMI become over 35 kg/m^2.

There is insignificant influence of infertility type and infertility duration on pregnancy rate and that consistent with study of Metello *et al.*, 2019 which could be due to the homogenous data of our study owing to inclusion and exclusion criteria that could help us to minimize demographic effect on our result .

Moreover; there is insignificant influence of cause of infertility on pregnancy rate, this can be attributed to the role of ICSI in overcoming almost all causes of subfertility and that consistent with study of Alasmari *et al*., 2018.

5.3.2. Reproductive Hormones in Pregnant and non-Pregnant Women

Basal FSH level was higher in pregnant women than non-pregnant one . Women under 35 years had a good quality oocytes in most cases, and FSH one of the predictor markers of the number of follicles, which can be translated to pregnancy rate.

Gupta *et al*., 2017 and Li *et al*., 2022 mentioned that there is insignificant hormonal differences in young women, where the majority

of the recruited women in our study were among young group. But it is predict the quality of oocytes and formation of good grade embryos in older women.

About LH, basic E2, E2 at day of hCG trigger and prolactin effect on pregnancy rate , there was insignificant difference between pregnant and non-pregnant women and that agreed with Sahmay *et al.*, 2012, Zhang,W., *et al*., 2019 and Kamel *et al.*, 2018 studies respectively (tab. 4-6).

5.3.3. Serum and Follicular Fluid Thyroid Hormone Level Difference between Pregnant and non-Pregnant women

The American Society for Reproductive Medicine (2015) recommended that the level of TSH should not be above 2.5 mIU/L before entering the in vitro fertilization (IVF) program (Pfeifer *et al.*, 2015).

So that there were many studies demonstrate the effect of TSH level on pregnancy rate and almost all compare between TSH level less than or equal 2.5 mIU/L and more than 2.5 mIU/L in euthyroid female.

Although our study highlighted that all patients have serum and follicular fluid hormone levels within the normal range, the current study result shown that there was a significant influence of serum TSH on pregnancy rate with a p-value=0.01 with serum TSH level of pregnant women significantly higher than non-pregnant one ($P<0.05$).

Our study was agree with Xia *et al.*, 2022 study who demonstrate that the higher implantation but miscarriage rates of IVF/ICSI may be associated to upper higher normal serum TSH levels.

Zhang, Y., *et al*., 2021 mentioned that a slightly but significantly higher live birth rate was observed in the high-normal TSH level and then they mentioned in another study at 2022 that high-normal TSH levels did

not have adverse effects on clinical and obstetric outcomes when compared with low-normal TSH levels.

While Jin *et al.*, 2019, Cai *et al.*, 2019 and Zhang, Y. *et al.*, 2021 shown no significant difference in the clinical pregnancy rate and the miscarriage rate in euthyroid women underwent IVF/ICSI with serum TSH ≤2.5 mIU/L and those with serum TSH >2.5 mIU/L.

Safarian *et al*., 2019 mentioned that levothyroxine treatment might increase pregnancy rate in women underwent ART, especially if the serum TSH level is more than 2.5 mIU/L with thyroid autoantibody or >4.0 mIU/L in general, considering that subclinical hypothyroidism is with TSH >4.0 mIU/L.

It seems that the study populations, different thresholds of TSH levels, and treatment strategies play vital roles in explaining the discrepancies.

Also this may be due to the development of IVF/ICSI therapy and the improvement of embryo culture system, which improves the possible adverse effects of elevated TSH either on oocyte, fertilization or implantation.

5.3.4. Comparison between Pregnant and non-Pregnant Groups Regarding the ICSI Outcome

In spite of a relatively slight increment in median of the total oocyte number, MII oocytes and grad A embryo in pregnant women than non-pregnant but that made insignificant association with pregnancy rate taking into account that all women were good or high responder and that could be explained by the recent studies including Li *et al*. 2020 and Leijdekkers *et al*. 2020 studies which indicate that the majority of the implantation success was determined by the chromosomal status, and the embryo aneuploidy rates appeared to be unrelated to the number of retrieved

oocytes, but women with higher numbers of oocytes or embryos were observed to have a higher absolute number of euploid embryos.

There is a significant difference in pregnant and non pregnant women regarding grade C embryos, p- value=0.02 with a median in pregnant women more . Sang *et al*., 2021, explained the higher percentage of malformations found in the bad-quality embryos due to a higher percentage of apoptotic features and chromosomal disorders and that have been reported to be the causes of embryonic arrest, and pre-implantation embryonic lethality, therefore; result in ICSI failure (Aldemir *et al.*, 2020).

So that discrepancy in the current study might be due to higher oocyte number in pregnant women which result in more embryos in different grades and so embryologist selection of a good quality embryos and their transfer might be the cause of ICSI success (Kim *et al*., 2021).

5.4. The Correlation of Serum (TSH, T4 and T3) and Follicular (TSH,T4 and T3)

The study result shown a significant positive connection between serum and FF-TSH levels in a p-value equal to 0.01, representing that the majority of TSH identified in follicular fluid seem to be derived from peripheral blood and enter follicles through theca interna cells.

Moreover, the study distinguished significantly higher concentrations of means of T3 in follicular fluid than in serum and T4/T3 ratio was much lower in follicular fluid than in blood (38.7% vs. 15.7%), which supports the presence of an ovarian 5′-monodeiodinase system in follicular fluid capable of generating T3 (ovary- generated T3) by outer ring de-iodination of T4 which consistent with Cai *et al*., 2019 study.

5.4.1. The Correlation of Thyroid Hormones Level with Reproductive Hormones

The positive correlation of S.T4 and FF.T4 with FSH which is consistent with the study of Safaryan *et al.,* 2019 and Iancu *et al.,* 2022 and that could be explained by; the essential role of thyroid hormones to have a synergistic effect with follicle-stimulating hormone, and in amplifying the effects of gonadotrophins and explain the essential role of THs in serving as a beneficial function in follicle growth, follicular development and ovulation.

The positive correlation of FF.T3 and E2 at day of hCG trigger indicate that FF.T3 increase granulosa cell proliferation while lowering apoptosis when combined with FSH. The interaction between T3 and gonadotropic hormones also prevents theca cells from producing too many androgens and promotes aromatization, which causes granulosa cells to produce more estrogen and that agreed with Silva, J., *et al.* 2018 study.

The positive correlation of FF.T4 with AMH could be clarified by granulosa cells express THs transporters and receptors, THs deficiency affects follicle development and results in atresia, which translate into lowers AMH levels (Benvenga *et al.*, 2021).

5.4.2. The Correlation of Thyroid Hormones Level with ICSI Outcome

The positive significant correlation of S.TSH and GV oocytes made the S.TSH as an obstacle in front of oocyte maturity but at the same time it didn't affect pregnancy rate and so Li *et al.* 2021 mentioned . The positive significant effect of FF.THs with total oocyte number and maturity (MII oocytes) in addition to 2PN fertilization could be explained by the presence of both T3 and T4 in the FF, making a direct action on the oocyte was conceivable and the widespread expression of thyroid

receptors (TRs) on the oocyte, granulosa, ovarian stroma, and cumulus cells (Rosales *et al.*, 2020). It has been established through in-vitro experiments in rats that THs stimulate the development of preantral follicles and thus its maturation. Therefore that significant effect might be a key factor in predicting the success of ART which could be gathering by: embryo quality, a higher mean number of retrieved oocytes, MII oocytes, blastocyst formation that found in the successful pregnancy group rather than in the implantation failure group. According to Cai *et al*.2021 and Benvenga *et al.* 2021 studies, inappropriate thyroid function in subfertile women may be an indication that they are unable to perform essential reproductive processes such as ovulation, fertilization, and also affect oocyte quality.

5.5. Binary Logistic Regression for Thyroid Function Test in Pregnant Women Against non-pregnant Women

Increase S.TSH within specific limit associated with success pregnancy about three times when compared with failed group and that was in accordance with the results of this study.

5.6. ROC Curve of Serum and Follicular Thyroid Function Test for Pregnant in Contrast to non-pregnant women

According to ROC curve which identify that only S.TSH had a significant influence on pregnancy rate with area under the curve equal to 0.7 and cut-off value equal to 1.5 which is the best predictor of pregnancy with sensitivity of 80% and specificity of 72% and that correlate with the result of Repelaer Van Driel-Delprat *et al.*, 2021 which they found that the best clinical pregnancy outcomes was when S.TSH cut-off value equal to 1.5 and less than 3 and they found that the area under the curve for S.TSH in clinical pregnant women was 0.5.

Chapter Six

Conclusions and Recommendations

Conclusions and Recommendations

6.1. Conclusions

1. Serum TSH was considered as predicting factor of pregnancy success with cut-off value equal to 1.5. and increase in S-TSH within specific limit associated with success pregnancy about three times when compared with failed group.
2. Serum TSH had a significant correlation to follicular fluid TSH so it might no need for measuring follicular fluid TSH as it is more invasive therefore can depend on S.TSH instead.
3. Follicular fluid T4 had a significant positive correlation with MII and 2PN. Besides FF.T3 had a significant positive correlation with oocyte number, MII, 2PN and embryos quality.
4. Follicle stimulating hormone levels were found to had a positive associations with S.T4 Plus FSH and AMH levels were found to have a positive associations with FF.T4. In addition to E2 levels were found to have strong associations with FF-T3.

6.2. Recommendations

1. A study including a large number of patients in multicenter is needed for further evaluation about the role of serum and follicular fluid thyroid hormones levels in predicting the outcome of ICSI cycles.
2. Study of women with hypo/hyper thyroidism with treatment and its effect on ICSI outcomes.
3. A study including patients with different ages, BMI and ovarian reserve.
4. A study of the effect of thyroid hormone levels in fresh and frozen embryo transfer.
5. A study of the role of thyroid hormones on ICSI outcomes using different COH protocols and different oocyte final maturation treatment.

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الخلاصة

الخلاصة

الخلفية العلمية: للهورمونات الدرقية دور فاعل في أجهزة الجسم جميعها تقريبا بما في ذلك الجهاز التناسلي ولذلك فأن لها دوراً مهماً في الأخصاب والحمل .

ومن جهة اخرى فأن قصور الغدة الدرقية مرض شائع في النساء في عمر الانجاب وقد يؤدي ذلك الى ضعف في الخصوبة ومشاكل في الحمل سواء عن طريق الحمل الطبيعي او بالتقنيات المساعدة على الانجاب.

حلت التقنيات المساعدة على الانجاب العديد من المشاكل المتعلقة بعدم القدرة على الانجاب عن طريق الاخصاب خارج الجسم لكن نسب النجاح لا تزال غير مرضية ولذلك فمن المهم دراسة أي عامل قد يؤدي الى تحسين النتائج.

الهدف من الدراسة: هو فحص العلاقة بين مستويات الهرمونات الدرقية في مصل الدم وفي السائل الجريبي و نتائج عمليات الحقن المجري.

صممت الدراسة كدراسة مستقبلية ، وقد حصلت على موافقة لجنة أخلاقيات البحث المحلية في كلية الطب / جامعة بابل.

أُشرك في الدراسة خمسون زوجًا يعانون من عدم القدرة على الانجاب بعد الخضوع لمعايير الشمول كما أُجريت الدراسة في مركز علاج العقم واطفال الانابيب في مستشفى الكفيل التخصصي في كربلاء المقدسة خلال الفترة من ايلول ٢٠٢١ إلى ايلول ٢٠٢٢ .

أُخضع كلا الزوجين للتقييم من أطباء المسالك البولية واطباء النسائية. اعتمد التقييم على الفحص الطبي والتاريخ المرضي فضلا عن الفحوصات المختبرية لتقييم الخصوبة. وقد أُخذت الموافقة الشفوية المسبقة من جميع الأزواج المشاركين في هذه الدراسة.

في يوم سحب البويضات، جُمع السائل الجريبي بعد عملية جمع البويضات واستخدم جهاز الطرد المركزي لفصل الخلايا عنه ثم جُمد عند درجةْ 80-حتى وقت

تقييم الهرمونات الدرقية بواسطة ELISA Kit

بعد جمع البويضات أُزيلت عنها الخلايا الجريبية كيميائيا و ميكانيكيا وقُيّمت تحت المجهر من حيث العدد والنضج والشكل .

ثم أُجري الحقن المجهري وبعدها قُيَّم مدى ارتباط نتائجه بمستويات الهرمونات الدرقية في مصل الدم و السائل الجريبي لاحقا.

النتائج: أظهرت النتائج أن الهرمون المحفز للغدة الدرقية في الدم كان ذا علاقة طردية في التنبؤ بحدوث الحمل بقيمة $p = 0.01$ وبمتوسط أعلى نسبيًا في النساء الحوامل منه في غير الحوامل (٢,١٣ ± ٠,٩٣ مقابل ١,٥٣ ± ٠,٦٧).

أضف إلى ذلك، فإن الهرمون المحفز للغدة الدرقية في مصل الدم كان له ارتباط كبير بالهرمون المحفز للغدة الدرقية في السائل الجريبي ($r = 0.33$ و$p = 0.0.$)

و فيما يخص علاقة الهرمونات التناسلية بالهرمونات الدرقية ، وجد أن لمستويات الهرمون المحفز للجريب ارتباطات إيجابية مع هرمون الغدة الدرقية رباعي يوديد الثيرونين في مصل الدم $r = 0.32$ و$p = 0.02$

وجد أن لمستويي الهرمون المحفز للجريب وهرمون مضاد موللیري ارتباطات إيجابية مع هرمون الغدة الدرقية رباعي يوديد الثيرونين في السائل الجريبي $r = 0.31$ $p = 0.024$بالإضافة إلى$r = 0.35$ ، $p = 0.01$ على التوالي.

فضلا عن ذلك، وجد أن لمستويات هرمون المودق علاقة ايجابية قوية مع هرمون الغدة الدرقية ثلاثي يوديد الثيرونين في السائل الجريبي.

وقد كان لهرمون الغدة الدرقية ثلاثي يوديد الثيرونين في السائل الجريبي ارتباط إيجابي كبير مع عدد البويضات الكلي وعدد البويضات الناضجة ونسبة الاخصاب بقيمة معنوية اقل من ٠,٠٥

فيما يتعلق بالانحدار اللوجستي للهرمون المحفز للغدة الدرقية في مصل الدم وجد ان نسبة الارجحية تساوي 2.64وقيمة $p=0.00$

واخيرا فيما يخص المنحنى المميز لاداء المستقبل وجد ان السائل المحفز للغدة الدرقية

في مصل الدم له اعلى قيمة تحت المنحنى بما تساوي 1.5 وقيمة حساسية تساوي 80% و قيمة خصوصية تساوي 72%

الخلاصة: خلصت الدراسة الحالية إلى أن الهرمون المحفز للغدة الدرقية في الدم يعد عاملا للتنبؤ بنجاح الحمل بقيمة حدية تساوي ١,٥. و انه كان أعلى بثلاث مرات في النساء الحوامل من غير الحوامل.

وزارة التعليم العالي والبحث العلمي

جامعة بابل ـ كلية الطب

فرع الفسلجة والفيزياء الطبية

تأثير الهرمونات الدرقية في مصل الدم والسائل الجريبي على نتائج الحقن المجهري للنطف في سايتوبلازم البويضات

رسالة مقدمة الى

مجلس كلية الطب- جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير

في العلوم/ الفسلجة الطبية

من قبل

نور ناظم سوادي عبود

بكالوريوس طب وجراحة العامة ،٢٠١٤

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2023 ميلادية

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