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Investigation of some biochemical markers and some elements in infertile women

A Thesis
Submitted to the Council of the College of Science, University of Kerbala in Partial Fulfillment of Requirements for The Degree of Master of Science in Biochemistry
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صدق الله العلي العظيم

سورة الشورى الآية : (49 – 50)

Dedication

To my parents

To my sons and husband… (Khalid)

To anyone who helped me

In this thesis.....

Inam Joudah Radhi

Certification

We certify that this thesis (Investigation of some biochemical markers and some elements in infertile women) was prepared under our supervision at the Department of Chemistry, College of Science, University of Kerbala, as a partial requirement for the degree of master of science in Biochemistry .

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Summary

Background: Infertility is a disease, usually of the reproductive system, defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse. Infertility has been increased in the last few years. Infertility in Iraq cannot be considered health problem related to the deforming of uterus or infections of the fallopian tube, however, it may be a result of the environmental , social, and economic causes.

Objective: The objectives of this study were : to investigate whether the biochemical changes in some parameter can affect the women infertility and to predict the value of Histidine-rich glycoprotein (HRG), inhibin B, Anti-Mullerian hormone (AMH), Copper (Cu), Iron (Fe), Zinc (Zn), and Magnesium (Mg) in infertile women.

Subjects and Methods: Thirty-three infertile women were enrolled from women clinic and twenty-two healthy fertile women were enrolled as a control group. Their ages range from (15 to 44) years. HRG, inhibin B, and AMH concentrations were measured in sera using enzyme immune sorbent assay (ELISA) technique. Trace elements Cu, Fe, Zn, and Mg were determined by spectrophotometric methods. The experimental work was performed in AL-Hussein Teaching Hospital of Kerbala from October 2016 to April 2017.

Results: The results of biostatistical analysis revealed a significant ($p = 0.05$, $p = 0.004$) decrease in the concentration of inhibin B and Zn respectively in the sera of infertile women compared with control group and a significant ($p< 0.000$) increase in concentration of Mg in sera of infertile women compared with the control group.

Follow-up examinations four months after receiving treatments revealed a significant increase in serum HRG concentration ($p=0.01$) and a significant decrease in serum AMH concentration ($p=0.04$) among infertile

women. Pelvic ultrasonography showed a significant ($p = 0.000$) increase in the number and the size of oocytes in both left and right ovaries infertile women.

Furthermore, the results showed significant ($p < 0.05$) decrease in concentration of Cu and Fe in infertile women whose their ages ranges from (15 to 30) years old compared with infertile women their ages ranges from (31 to 44) years old.

Depending on the family history of infertility, the results showed a significant ($p=0.03$, $p=0.02$) decrease in concentration of HRG and Fe respectively in sera of infertile women with family history group compared with infertile women without family history. Also, the results revealed a significant ($p=0.02$) increase in the concentration of Mg in sera of infertile women with family history group compared with infertile women without family history group.

Conclusion: Infertile women are associated with the decrease level of inhibin B and Zn whereas there is an increase in the magnesium level. Also, inhibin B is considered a better marker for predict infertility than AMH.

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List of abbreviations

Terms	Definitions
Abs	Absorbance
ACTH	Adrenocorticotrophic releasing type 2
AFC	antral follicular count
AMH	Anti-Müllerian hormone
ART	Assisted Reproductive Technology
BMI	Body mass index
BMPs	Bone morphogenetic proteins
5-Br-PAPS	2-(5-Brom-2-pyridylazo)-5-(N-propyl-N sulfo propyl amino)-phenol
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
COS	Controlled Ovarian Stimulation
CTMA	cetyl trimethl ammonium bromide
Cu	Copper
Di-Br-PAESA	3, 5-Dibromo-2-pyridylazo-N-ethyl-N-3-sulphopropyl aniline
DW	Distilled water
E1	Estrone
E2	Estradiol
ELISA	Enzyme Linked Immuno Sorbent Assay
Fe	Iron
FF	follicular fluid
FGF	fibroblast growth factor
FSH	Follicle-stimulating hormone
GEDTA	Glycol etherdiamine -N,N,N,N-tetra acetic acid
GETA	Glycol ether tetraacetic acid
Gn-RH	Gonadotropin-releasing hormone
hCG	human chorionic gonadotrophin

Terms	Definitions
hMG	Human menopausal gonadotropin
HRG	Histidine-rich glycoprotein
HRP	Horseradish Peroxidase
INH-B	Inhibin B
IVF	*in-vitro* fertilization
KDa	Kilo Dalton
L	Liter
LH	Luteinising hormone
LPD	The deficiency of luteal phase
Mg	Magnesium
mg	Milligram
MIS	Mullerian Inhibiting Substance
ml	Milliliter
mmole	Millimole
µl	Microliter
ng	Nanogram
^{o}C	Degrees Celsius
OD	Optical density
OHSS	Ovarian Hyperstimulation Syndrome
OS	Oxidative stress
PCOS	polycystic ovary syndrome
P-Value	Probability level of statistical
r	Correlation coefficient
ROS	Reactive oxygen species
S.E	Standard error
SHRP	streptavidin-horseradish peroxidase
SPSS	Statistical Package for the Social Sciences
TGF-β	transforming growth factor β
TMB	Tetramethylbenzidine
VEGF	vascular endothelial growth factor
WHO	World Health Organization
Zn	Zinc

Chapter one Introduction and Literature Review

Introduction:

Infertility is a disease; of the reproductive system, defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse [1]. The inability to become pregnant results in important affecting and profitable tolls, among that artificial nervousness and sadness are more common in infertile couples than in fertile couples. Also Assisted Reproductive Technologies ART are accessible to care for infertility, but these are pricey. The credit of changeable hazard factors for infertility could guide to economical and helpful interventions expected at preclusion [2].

Due to wars in Iraq, around 2003, the Iraqi environment suffered from acts of profanation. A large number of injuries and deaths were caused by the destructive chemicals and radioactive materials. The people who survived these devastating incidences either suffered from cancer or infertility. However, there are only a few studies that have examined the postwar examined specifically on infertility [3].

Infertility affects from 80 million to 168 million people in the world today. Approximately one out of ten couples experience primary or secondary infertility [4]. It is a global complexity that affects approximately 15% of all couples, and about 33% of the cases of infertility are mainly credited to the womanly [5]. In the last thirty years, Iraq exposed several instability crises causes exasperation in many health problems. One of these problems was the fertility status of many people in all of the country. Few studies deal with this health problem in spite of the mental, communal and fiscal proportions of this subject [6].

Histidine-rich glycoprotein (HRG) is a multi-domain protein involved in the coagulation, angiogenesis and immune response, all of them are important for the institution of pregnancy [7].

Inhibins are dimeric polypeptide hormones that belong to the transforming growth factor β (TGF-β) superfamily. Inhibin B, a paracrine ovarian and testicular regulator is secreted by the granulosa cells of the ovary has multiple paracrine effects on the uteroplacental unit, instead of promising marker poverty for both male and female [8].

Anti-Müllerian hormone (AMH) is a glycoprotein produced by the granulosa cells of preantral and small antral follicles that participates in folliculogenesis instruction [9]. It is also called Mullerian Inhibiting Substance (MIS), that is a homodimeric glycoprotein from the TGFβ family. It plays a major role in cell growth and differentiation [10]. It is widely accepted that the reduction of AMH levels in serum is the first indication for the decline in the follicular reserve of the ovaries. It can be measured in the blood at any time in the menstrual cycle due to its stability [11] and an extrapolative factor of a reaction to the ovarian stimulation with gonadotropins during the stimulation of ovulation [9].

Trace elements participate with a significant role in the anticipation of many age-related diseases, and in maintaining normal immune and cognitive functions [12]. Trace minerals exist in cells and tissues of the human body in a variety of chemical combinations, and in feature concentrations, depending on the trace mineral addicted and the tissue in which the trace mineral is metabolized [13]. Copper is crucial to human life and health that plays a key physiological role as the prosthetic element of more than a dozen precise copper proteins which has a significant role to play in haem and collagen production [14].

According to a recent study in which women who took iron supplements had nearly half the risk of developing ovulatory infertility compared with women who did not use iron supplements [15].

Zinc is an important trace metal that the body uses to keep hormone like estrogen, progesterone and testosterone levels steady during the whole menstrual cycle. It is especially important during stages 2 and 4 of a woman's cycle [16].

Magnesium is the most abundant divalent cation in the intracellular compartment, where it serves as a co-factor in a number of biological systems [17]. Mineral elements, including copper, iron, zinc, and magnesium are required by the body in modest amounts for the maintenance of health and for the development of optimal physiological function [18].

Gonadotrophins are hormones (luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that can be given by in injections to stimulate a woman's ovaries to produce follicles, which contain oocytes [19]. Gonadotrophin treatment is connected to a higher rate of multiple pregnancies, particularly in women with anovulatory infertility, and is considerably costly [20].

Letrozole, an aromatase inhibitor, is regarded as a second line treatment option, particularly in women with clomiphene resistance. Letrozole prevents the conversion of androgens to estrogen, thus releasing the hypothalamic-pituitary axis from the negative feedback of estrogen, results in an increase of FSH secretion from the anterior pituitary [21].

1. Literature Review

1.1 Infertility

Infertility affects about 10-15% of reproductive age couples. Infertility, according to World Health Organization WHO, is defined as a disease of the reproductive system defined by the failure of clinical pregnancy after 12 months or more of regular unprotected sexual intercourse. Prevalence of infertile individuals is rapidly increasing globally. Female factors contribute 40-45% in etiology of infertility [22]. An evaluation also may be initiated former when the female partner is older than 35 years, because fertility rates decrease and spontaneous miscarriage and chromosomal abnormality rates increase with advancing maternal age [23]. Infertility left a woman's social burden although male infertility contributes to more than half of all cases of global barrenness which some regions of the world, such as in developing countries, the percentage of infertility could reach an average of 30% [24].

Every couple wants to bear a child but not all couples who wish to have a child will realize it without medicinal help. It is found that more than 70 million couples worldwide undergo from infertility and the majority of them live in developing countries [25]. In the last three decades, three arresting changes have occurred in infertility practice, namely the introduction of Assisted Reproductive Technology (ART), a marked increase in patient visits for infertility and an increase in the proportion of women over age 35 years looking for therapeutic consideration for infertility [26]. The frequency of infertility differs very much from one country to another, being 15% worldwide, > 30% in some developing countries, and 17.28% in manufacturing countries [27]. Numerous medical disorders can donate to infertility by causing injury to the fallopian tubes, interfering with ovulation and fertilization, or causing hormonal turbulence [5]. Unable or difficult to conceive is a physically and psychologically painful condition in a female's life [28].

The most important organs and structures in the reproductive system is the uterus, it is a vacant muscular organ and pear-shaped organ located between the bladder and lower intestine. It consists of two parts, the body and the cervix which is the lower portion of the uterus. It has a vessel opening into the vagina with an opening called the os, which allows menstrual blood to flow out of the uterus into the vagina and leading off each side of the body of the uterus into two tubes known as the fallopian tubes. Next to the closing stages of each tube is an ovary. Ovaries are egg-producing organs that hold 200,000 to 400,000 follicles [29].Fig. [1.1].

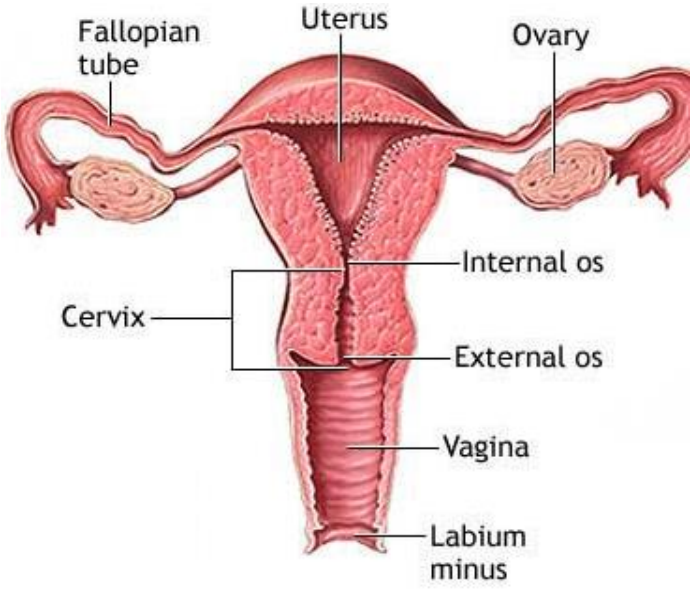


Fig.[1.1] The reproductive system of women [29]

1.2 Type of Infertility

Infertility is classified into two types:

1. Primary infertility

Primary infertility is a term used to describe a couple that has never been able to conceive after a minimum of 1 year of attempting to do so through unprotected intercourse [30] and as the absence of a live birth in a sexually active non-contraception woman [31].

2. Secondary infertility

Secondary infertility is usually defined as the inability to conceive despite exposure to pregnancy for one year (2 years in some epidemiological studies), after having conceived at least once before [32]. Secondary infertility owed to the failure to become pregnant or the failure to bear a pregnancy to a live birth following either a preceding pregnancy or a preceding gift to bear a pregnancy to a live birth [33].

1.3 Causes of female infertility

Female infertility can be caused by many different factors:

1.3.1 Disorders of ovulation

The (WHO) classifies ovulation disorders into three groups:

- **Group I**: hypothalamic pituitary failure (hypothalamic amenorrhea or hypogonadotropic and hypogonadism).
- **GroupII** hypothalamic-pituitary-ovarian dysfunction, predominately a product of polycystic ovary syndrome (PCOS). This is the cause of the enormous greater part of ovulation disorder.
- **Group III**: ovarian failure a result hypo-thalamic cause/body fat over 35 (Kg/m) Body Mass Index BMI will result in irregular menses, amenorrhea, or failure to ovulate and increase in pituitary hormones of FSH or LH will result in failure to ovulate [34].

1.3.2 Male factor

There are six main causes of infertility in males. The first one is abnormal sperm production or function. Sperm can be affected by repeated infections, genetic defects or undescended testicles [35]. Male infertility is often connected with abnormal semen analysis, but can also be idiopathic with a normal semen investigation and primary testicular failure which is the most common cause of male infertility [36].

Male factor infertility is assessed based on the following values [37]:

- underprovided sperm count (less than 10 million per milliliter; volume should be 1 -5 ml. of ejaculate)
- deficient sperm motility (over 60% should be motile and show purposeful forward movement).
- poor sperm morphology (more than 50-60% abnormal in form)

1.3.3 Combined female and male infertility

The main contributing factor for gonadal dysfunction and infertility in female and male patients with rheumatic diseases seems to be drug therapy [38]. Both men and women suffer from stress and over time, continued strain on the body from routine stress may lead to serious health problems, as well as depression and anxiety disorders [35].

1.3.4 Unexplained infertility

The diagnosis of unexplained infertility is given when the standard fertility evaluation is normal in both the man and the woman and depends on the number, nature, and quality of the tests used, and the interpretations made [36]. Couples with unexplained infertility may actually suffer from the subclinical expression of acknowledged causes of infertility that could be revealed by further testing or continued observations [39].

1.3.5 Recurrent miscarriage

Recurrent miscarriage defined as the loss of three or more consecutive pregnancies affects 1% of couples trying to conceive. It has been estimated that 1–2% of second - trimester pregnancies miscarry before 24 weeks of gestation[40] . Less than 5% of women experience two consecutive miscarriages, and only about 1% has three or more consecutive miscarriages. Recurrent miscarriage can be subdivided into primary and secondary recurrent miscarriage, potentially reducing the heterogeneity of this population [36].

1.3.6 Endometriosis

Endometriosis is a chronic condition characterized by the growth of endometrial tissue in areas other than the uterine cavity, most commonly in the pelvic cavity, including the ovaries and the endometrial deposits within the ovary that are known as endometriomas. Tubal damage can occur as a result of endometriosis also the symptoms include pelvic pain, painful periods, and pain during and after intercourse [41].

1.3.7 Uterine or cervical with the fallopian tubes causes

There are several congenital or acquired uterine disorders, which cause infertility in the females; the acquired uterine abnormalities that are responsible for female infertility include polyps, some types of fibromas, adenomyosis, and some endometrial disorders such as intrauterine adhesions [42]. At the top of the vagina is the neck or entrance to the uterus, called the cervix. Ejaculated sperm must travel through the cervix to reach the uterus and fallopian tubes; cervical mucus around the time of ovulation is normally thin and watery so that sperm can swim through. However, thick or poor quality cervical mucus can hinder the sperm and the sperm fertilizes the egg on its journey down the fallopian tube. A blocked or scarred fallopian tube may impede the egg's progress, preventing it from meeting up with sperm [43].

1.3.8 Oxidative Stress

Oxidative stress (OS) can be defined as the imbalance between the generation of oxidants and the concentration of antioxidants, in which the generated reactive oxygen species ROS are favored, resulting in harmful effects on the body [44]. ROS may act as key signaling molecules in physiological processes but at excess, uncontrolled levels may also mediate pathological processes involving the female reproductive tract. The role of OS has been demonstrated in many causes infertility, such as

endometriosis, polycystic ovarian disease, unexplained infertility, tubal infertility, and recurrent pregnancy loss [45]. Fig (1.3).

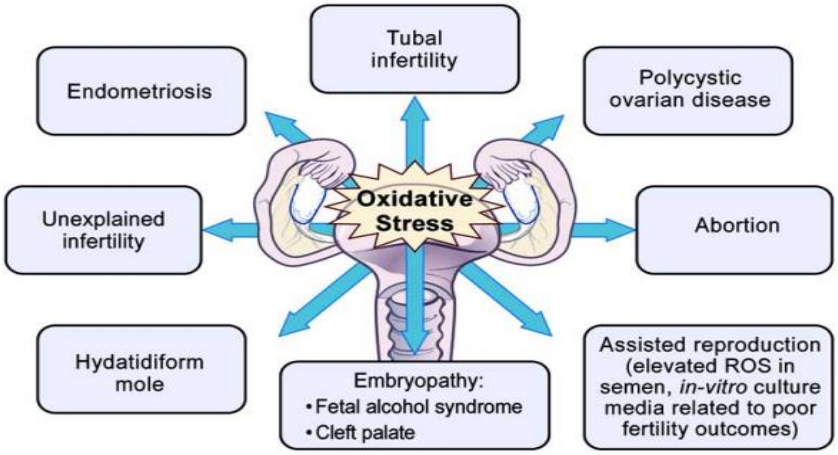


Fig. (1.2) The role of OS in female infertility [45]

1.3.9 Hormonal causes of female infertility

- **Ovarian Insufficiency**: The average age of menopause is 51, with some women having their last period in their forties and others later in their fifties. A cessation of ovulation prior to the age of 40 is rare and is usually referred to as premature ovarian failure [46].
- **Luteal Phase Deficiency:** the deficiency of luteal phase LPD progesterone synthesis and/or action is the leading cause of infertility or spontaneous abortion in cases of LPD [47].
- **Polycystic Ovarian Syndrome (PCOS):** PCOS is the most common endocrine disorder affecting women of reproductive age and is closely associated with insulin resistance, metabolic syndrome and future risk of developing diabetes and cardiovascular disease [46].
- **Thyroid disorders:**: Hyperthyroidism and hypothyroidism can disrupt the menstrual cycle and cause infertility [48]. Both hyper and hypothyroidism may result in menstrual disturbances. The most common manifestation is simple oligomenorrhea (decreased menstrual

flow). Anovulatory cycles are very common. Increased bleeding may occur, but is rare in hyperthyroidism [49].

- **Stress rises**: The inability to bear children is a very stressful situation and infertility can cause a multitude of adverse social and psychological consequences that included aggravated mental distress [50].

1.3.10 External causes of female infertility

1. Contraception

Contraceptives are extensively used, especially by young individuals whose reproductive years generally lie ahead of them. The types of contraception used vary with age, marital status, reproductive history, and race [39].

2. Sterilization

For reversal of contraceptive sterilization in women, several factors are important in determining whether fertility can be restored: the surgical method initially used, tubal site, length of tube remaining, and surgical skill in restoration. About 2 % of women used some form of periodic abstinence. Withdrawal, douche, foam, and suppositories were used by similarly small percentages of women [39].

3. Abortion

Abortion means the termination of pregnancy before the fetus reaches viability [51]. Abortion is frequently stated that a high proportion of women who have an induced abortion by dilatation and curettage are subsequently infertile [52].

4. Environment and Drugs

Currently, no reliable estimates can be made of reproductive risk from environmental factors. Until recently, little attention was paid to environmental and drug-induced infertility and sub infertility. However, four health hazards ionizing radiation, lead, ethylene oxide, and dibromochloropropane are regulated in part because of their effects on

the reproductive system. Possible environmental hazards include chemical agents; physical agents such as altitude, temperature, and radiation; and personal habits such as smoking, alcohol consumption, use of drugs (both therapeutic and nontherapeutic), and eating patterns [39].

5. Smoking

In humans, evidence suggests that smoking has a deleterious effect on menstrual cyclicity, oocyte production, and tubal function [40]. Smoking has been found to have an adverse effect on fertility and conception as well as most phases of the development of the child in the womb and on post-natal survival. Some of the negative reproductive consequences associated with smoking include quicker depletion of ovarian follicles, conception delay, increased risk of spontaneous miscarriage in both natural and assisted conception cycles, and increased risk of birth defects [41].

6. Spinal Cord Injury

The outlook for paraplegic women is often better. Problems resulting from spinal cord injury include the inability to achieve an adequate erection, inability to ejaculate normally **[**39**].**

7. Female age

Female age is the most important determinant of spontaneous conception and treatment-related conception. Fertility begins to decline in females from the age of 30 years old, although the reduction in fertility is greatest in women in their late 30 years old and early 40 years old. The number of competent oocytes in the ovaries declines with increasing age. For women up to 25 years old the cumulative conception rate is 60% at six months and 85% at one year, but conception rates for women aged over 35 years old are less than half of this. Current recommendations state that women aged over 35 years old

should be classed as having advanced reproductive age and referred more promptly for early investigations and active treatment [41].

8. **Drugs**

Drugs are known to impact on fertility. Non-steroidal anti-inflammatory drugs, commonly used to treat pain or inflammation are known to inhibit ovulation. Cytotoxic chemotherapy drugs are also known to cause ovarian failure in some women. Recreational drugs such as marijuana can have an adverse effect on ovulation and cocaine appears to adversely affect tubal function [41].

9. Weight

Obesity is defined by an extraordinarily high Body Mass Index (BMI) is a measurement of body fat calculated from an individual's weight and height (weight in kg/height in $metres^2$ [41], in which the index is a reflection of body fat content. Around 1 out of 4 women are at least overweight as per surveys and studies. The rates are higher among women facing problems of conception [53]. Overweight as BMI between 25.0 and 29.9 kg/ m^2 [54].

The overweight of obesity is associated with decreasing pregnancy rates, increasing requirements for gonadotrophins and a higher miscarriage rate. These differences are evident at a BMI over 25. A high BMI is also associated with adverse pregnancy outcomes such as gestational diabetes and hypertension. [41].

10. Physical activity

Physical activity is known to be beneficial to general health. Most researchers conducted on physical activity and reproduction mainly focused on athletes and vigorous exercise. Evidence from the large Nurses' Health cohort study showed that an increase in vigorous but not moderate physical activity is associated with reduced relative risk of ovulatory infertility. Physical activity improves insulin sensitivity,

which in turn improves ovarian function and therefore the chance of conception [41].

11. Alcohol

Alcohol is teratogen and its consumption that has been reported to reduce fertility, although the level of consumption associated with risk is unclear, drinking one alcoholic drink per week has been reported to be associated with a reduced chance of conception, a prospective observational study of 124 women reported more than a 50% reduction in the probability of conception among participants who drank alcohol [41l.

1.4 Diagnosis of infertility

The most common examination for diagnosis of infertility is:

- couple's history and physical exam,
- semen analysis,
- basal body temperature charts and menstrual cycle mapping,
- cervical mucus evaluation,
- hormone assays
- post- immunologic evaluation coital test
- endometrial biopsy,
- hysterosalpingogram,
- laparoscopy,
- hysteroscopy,
- Hamster-egg penetration assay [39].

1.5 Treatment of female infertility [55]

Infertility can be treated with medicine, surgery, artificial insemination, or assisted reproductive technology. Many times these treatments are combined. In most cases, infertility is treated with drugs or surgery.

Doctors recommend specific treatments for infertility based on:

- Test results,
- How long the couple has been trying to get pregnant,
- The age of both the man and woman,
- The overall health of the partners,
- Preference of the partners.

1.6 Drugs used in the treatment of female infertility

Some common medicines used to treat infertility in women include:

1. Clomiphene is the current first-line infertility treatment in women with the polycystic ovary syndrome, but aromatase inhibitors, including letrozole, might result in better pregnancy outcomes [56].
2. Human menopausal gonadotropin or hMG (Repronex, Pergonal): This medicine is often used for women who don't ovulate due to problems with their pituitary gland. hMG acts directly on the ovaries to stimulate ovulation. It is an injected medicine [55].
3. Follicle-stimulating hormone or FSH (Gonal-F, Follistim): FSH works much like hMG. It causes the ovaries to begin the process of ovulation. These medicines are usually injected [55].
4. Gonadotropin-releasing hormone (Gn-RH) analog: These medicines are often used for women who don't ovulate regularly each month. Women who ovulate before the egg is ready can also use these medicines. Gn-RH analogs act on the pituitary gland to

change when the body ovulates. These medicines are usually injected or given with a nasal spray [55].

5. Metformin (Glucophage): Doctors use this medicine for women who have insulin resistance and/or PCOS. This drug helps to lower the high levels of male hormones in women **[**55**].**

1.7 Parameters under study

1.7.1 Histidine-Rich Glycoprotein

Histidine-rich glycoprotein (HRG) is a glycoprotein involved in fibrinolysis, coagulation, immunological response system and angiogenesis. It is produced by the liver and transported both as a free serum protein and also in granules in platelets; it blocks the anti-angiogenic effect of thrombospondin and also promotes cell migration and invasion by binding to plasminogen and plasmin [57].

HRG is present in the reproductive system as well as in the embryo. Little is known about the existence and function of HRG within the female reproductive tract [58].

In particular, the regulation of HRG function upon binding of Zn^{+2} or changes in environmental pH has become a recurring observation in recent years, given the multi-domain character of HRG that mediates interaction with a large array of different ligands [59]. The concentration of HRG in plasma is100-150 μg/ml, the concentration of HRG in plasma varies throughout life [36]. HRG is a multidomain protein consisting of 2 cystatin-like regions at the N-terminus (N1 and N2), a central histidine-rich region (HRR), 2 proline-rich regions (PRR1 and PRR2) flanking the HRR, and a C-terminal domain (C). Proposed carbohydrate attachment sites, disulfide bridges, binding partners of HRG, and the corresponding binding regions of HRG (ie, N1N2, HRR, or C) are depicted in the figure. Note that the binding sites for phospholipids, heparanase, fibrinogen, DNA, and certain complement components on HRG have not been characterized [60]. Fig. (1.3).

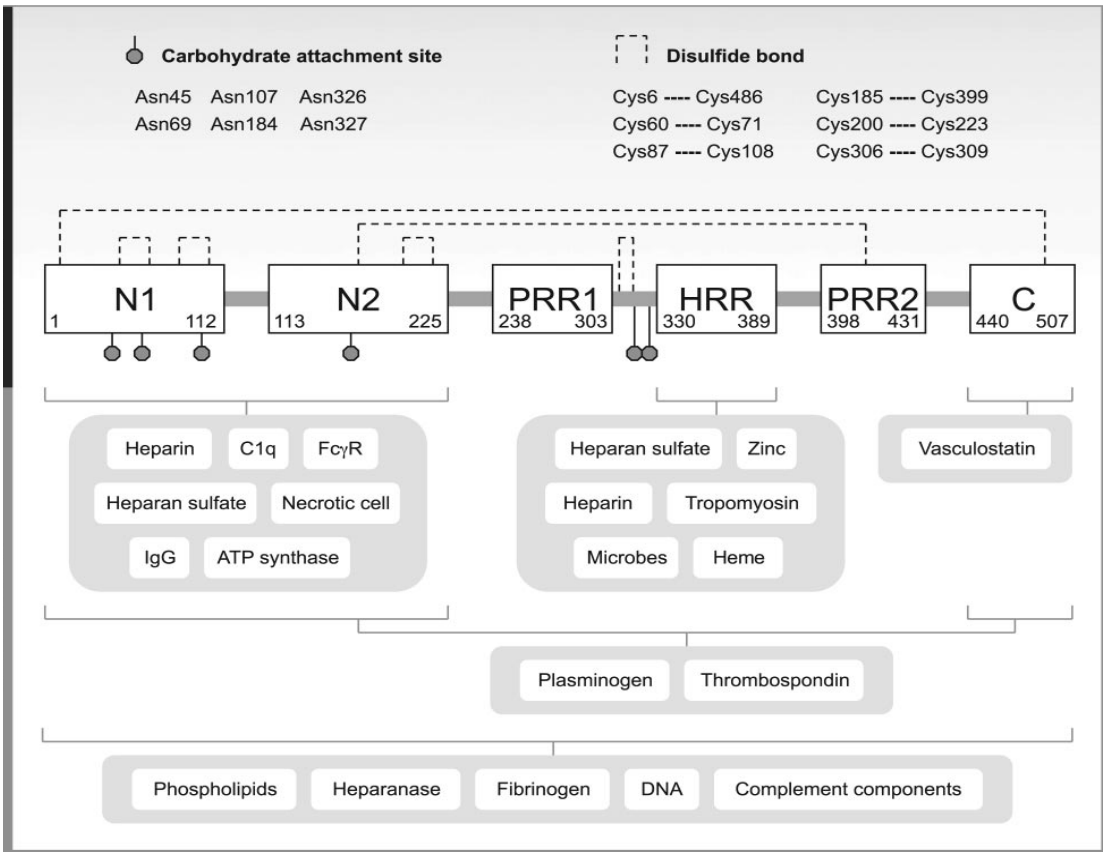


Fig. (1.3) Schematic representation of protein domains, posttranslational modifications, and proposed binding partners of human HRG [60]

1.7.2 Inhibin B

Inhibin B (INH-B) is a dimeric glycoprotein produced by the gonads with a recognized role in inhibiting FSH secretion ,and in fertile-aged women, its plasma concentrations are maximal in the follicular luteal transition phase of the cycle, rising 4 days after the FSH peak, implying that FSH stimulates the production of inhibin B. Among the etiologic factors proposed as cause of infertility (mechanical distortion of tuboovarian anatomy, abnormal serum and follicular fluid hormonal patterns, alterations in peritoneal fluid milieu and in the endometrial environment) on impairment of follicular development has a relevant incidence [61] .

Inhibin B is produced by granulosa cells of preantral and early antral follicles, and its levels are usually high during the follicular phase of the menstrual cycle and low during the luteal phase. The recent studies in normal-

ovulatory women have shown convincingly that inhibin B is predominantly secreted by granulosa cells of preantral and small antral follicles and hence its concentration increases during the luteo- follicular transition. Inhibin B level is highest during the mid-follicular phase and decline during the late follicular phase [62]. Inhibin-B is positively in combination with 17β-estradiol and with the number of oocytes, and it correlates with the pregnancy rate in patients receiving treatment with *in-vitro* fertilization IVF [63].

The primary endocrine role of inhibin B appears to be the regulation of gametogenesis via a negative feedback mechanism on the production of FSH by the pituitary gland. Inhibin B may also exert local paracrine actions in the gonads [8]. Inhibin is acting in a classical endocrine manner and negatively regulates the activin stimulation of FSH. Inhibin is binding to the same receptor Adrenocorticotrophic releasing type 2 (ACT RII) inhibits the secretion of FSH and LH. The preliminary evidence suggests that activins appear to stimulate the gonadotrophin-releasing hormone (GnRH) release in the hypothalamus [64]. Fig. **(**1.3).

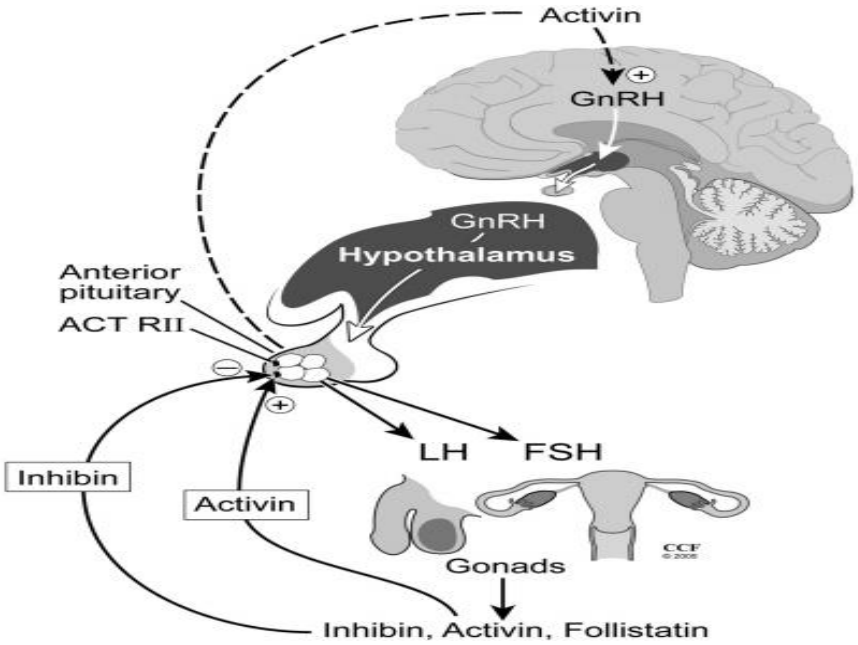


Fig. (1.4) Schematic mechanism of regulation of inhibin and activins on gonadotrophin secretion [64]

1.7.3 Anti - Mullerian Hormone

AMH is a glycoprotein hormone, with a molecular weight of 140 kDa [65], it also referred to as Müllerian -inhibiting substance (MIS), is a member of the belonging to the TGF-β growth factor superfamily, which includes more than 35 structurally related peptides, including activins, inhibins, bone morphogenic proteins (BMPs) and growth differentiation factors [66]. In women, AMH is produced by granulosa cells in primordial preantral and small antral follicles and participates in folliculogenesis regulation [9]. AMH levels are thought to be stable throughout the menstrual cycle [67].

TGF- family ligands are translated as dimeric precursor proteins comprising two polypeptide chains, each contains a large N-terminal pro-region and a much smaller C-terminal mature domain, which must undergo cleavage at di-basic or mono-basic sites located between the two domains to generate the mature protein, and processing of a TGF- family ligand that was shown for a homodimeric precursor as was the case for AMH, but heterodimeric precursors also exist for other TGF- family members [68]. Fig. (1.4).

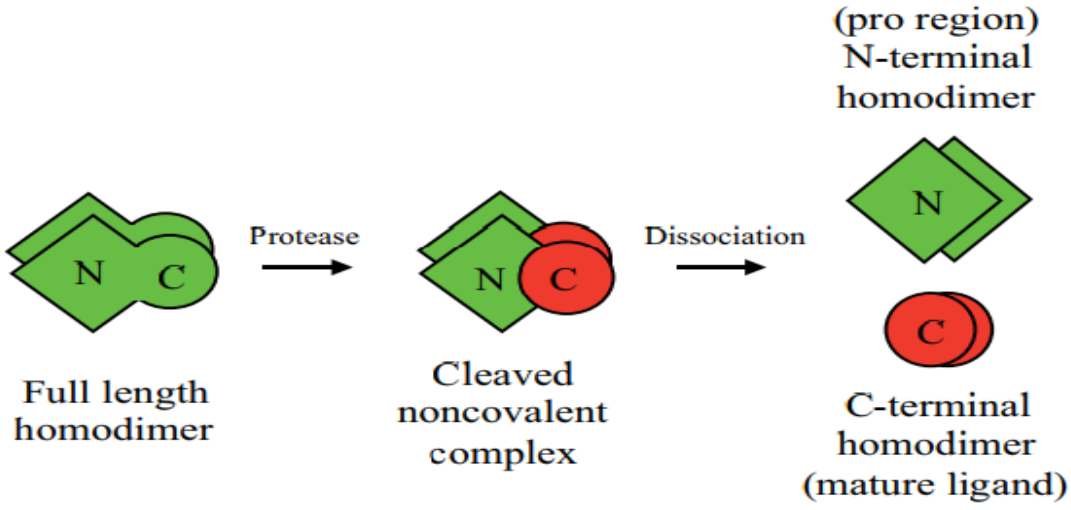


Fig. (1.5) Schematic diagram showing processing of a TGF- family ligand [68]

Recently wide-scale research studies focused on the usefulness of this hormone as a clinical marker of the ovarian reserve and a predictive factor of a response to the ovarian stimulation with gonadotropins during the stimulation of ovulation [9]. AMH concentrations are slightly lower during the luteal phase compared with the follicular phase of the menstrual cycle of women. Antral follicle count (AFC) is the number of follicles growing during the ovulation wave. AFC was positively associated with AMH concentrations levels and AMH is a good indicator for high, medium, and low AFC [69].

AMH is secreted by granulosa cells of growing follicles up to the small antral stage (≤8 mm). It has been suggested that AMH inhibits the growth of resting follicles and inhibits the effect of FSH on follicular growth [70].Fig. (1.6). .

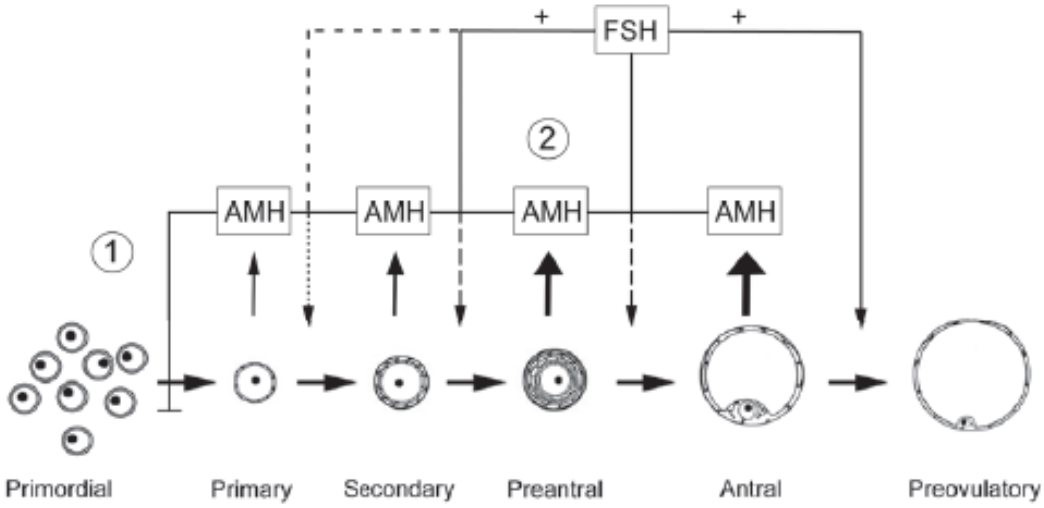


Fig. (1.6) The secretion and the possible role of AMH in the ovary [70]

1.7 .4 Trace elements

Trace elements [Copper (Cu), Selenium (Se), Zinc (Zn), Iron (Fe), Molybdenum (Mo), Manganese (Mn), Cobalt (Co), Chromium (Cr), and Iodine] are inorganic substances that are vital for sustaining life, are required in small amounts every day (generally less than 100 mg/day) [71] .

Trace elements have important functions in the human body. They are required in low concentrations and essentially they serve as very important cofactors for many antioxidant enzymatic reactions [72]. Trace metals have been shown to influence hormones at several levels of action, including hormone secretion and activity and target tissue binding. Similarly, hormones have been shown to influence trace metal metabolism at several levels of action, including excretion and transport of trace metals [73].

Trace elements are crucial for maintaining human health, as well as for preventing several health problems. Alteration of normal homeostasis of trace elements may adversely affect biological processes leading to many diseases processes [74]. Recently, it has been found that trace elements are also essential for life activities and they are closely related to female infertility abortion and fetal growth [14].

1.7.4.1 Copper

Copper (Cu) is an important trace element for numerous metalloenzymes and metalloproteins which are involved in energy or antioxidant metabolism [75]. Copper is one of the most common mineral imbalances contributing to infertility involves copper, both an excess and ⁄ or a deficiency can interfere with pregnancy, fetal health, and development. Copper can be stored in excessive amounts in cells, organs, and tissue. Women can accumulate copper from taking estrogen hormone replacement or the oral contraceptive pill [76]. Copper is essential to human life and health. It plays a key physiological role as the prosthetic element of more than a dozen specific copper proteins and has a significant role in haem and collagen production and function. The role of copper in human reproduction has not been much investigated. That low plasma Copper in the infertile women operates by impairing the structure and function of the supporting collagen in graaffian follicle or by direct inhibition of ovum transport through fallopian tubes [14].

1.7.4.2 Iron

Most women know the importance of getting enough of iron (Fe) once they are pregnant. Studies have shown that women who do not get sufficient amounts of iron may suffer anovulation (lack of ovulation) and possibly poor egg health, which can inhibit pregnancy at a rate 60% higher than those with sufficient iron stores in their blood [77].

The Iow levels of iron often confirmed by a low blood serum ferritin result. It has been directly linked to infertility despite a normal hemoglobin level and no symptoms of anemia. The low iron levels have been directly linked to low thyroid function and altered thyroid hormone levels may be linked to infertility and miscarriage; also women are generally low in iron due to menstruation and pregnancy [76]. The trace element Fe plays a key role in supplying oxygen for tissues where it is involved in the heme structure of hemoglobin [12]. Indirect evidence suggests that the direct effect of iron is probably related to its direct deposition on the hypothalamic-pituitary axis and the female reproductive system, while direct evidence suggests that its indirect effect is mostly attributed to the iron-induced OS; However, there are limited data evaluating the pathophysiology of iron-induced compromised fertility, in which there is no clear discrimination between direct or indirect iron effects [78],

1.7.4.3 Zinc

Zinc (Zn) is just one component that works with more than 300 different enzymes in the body to regulate many metabolic pathways. In women, zinc plays a vital role in many key reproductive health areas including egg production, maintaining proper follicular fluid levels and hormone regulation. Zn is an important trace metal that the body uses to keep hormone like estrogen, progesterone and testosterone levels stable throughout the entire menstrual cycle. It is especially important during stage 2 and 4 of a woman's cycle [16].

Zinc plays a role in ovulation and the menstrual cycle, which means that zinc deficiencies can make it harder to get pregnant. Low zinc levels have been linked to hormonal imbalances, which can cause ovarian function problems, irregularities in menstruation or even anovulation (in which women don't ovulate) [79].

Low and deficient zinc levels in women may cause deterioration in oocytes and in severe cases causes anovulation. [76]. Zinc is co-factor for more than 80 enzymes (including many so-called zinc finger proteins) involved in DNA transcription and protein synthesis and given the obvious importance of those processes in the fertilization and sustenance of an embryo. It is hardly surprising that zinc would be a crucial nutrient to consider in relation to fertility[80].

1.7.4.4 Magnesium

Magnesium (Mg) is the fourth most abundant essential mineral and the second most abundant intracellular divalent cation. It is involved as a cofactor for over 300 metabolic reactions in the body [81]. Adrenal insufficiency and prolonged stress impair the body's ability to utilize magnesium and toxic heavy metals such as cadmium block the absorption and utilization of magnesium [76]. The essential requirement of magnesium for sex hormone production and function underlies the importance of magnesium in infertility. it is known that estrogen receptor binding is a magnesium-dependent process and magnesium modulates FSH binding to receptors on the ovary [82].

Some studies have noticed a link between low magnesium deficiency and female infertility. Other functions of magnesium in the body are: magnesium ensures proper blood supply to the uterus and is also important for the production of progesterone, a hormone that's important in the menstrual cycle and is sometimes called "the hormone of pregnancy"[83].

The low magnesium may be a culprit if women are having difficulty getting pregnant and lead to infertility. Also, magnesium deficiency can cause spasms in a woman's fallopian tubes, preventing egg implantation [84].

1.8 The aims of the study

1. To investigate whether the biochemical changes in some parameters can affect the women infertility.
2. To predictive the value of inhibin B, Anti-Mullerian hormone, and Histidine-rich glycoprotein in infertile women.
3. To increase the knowledge about the causes of infertile women.
4. To show the correlation between the parameters under study.
5. To evaluate the clinical significance of some trace elements, Cu , Fe, Zn and Mg in infertile women.
6. To study the effect of medication (letrozole, and Gonadotropine) on ovulation induction and its relation with glycoproteins by follow up the infertile women**.**

Chapter two
Materials and Methods

2. Materials and Methods

2.1. Materials and Equipment

The materials and equipment used are summarized in table 2.1.

Table 2.1. Materials and Equipment

No	Instrument	Company
1.	Enzyme-linked immunosorbent Assays (ELISA)	Bio Tek Instruments 217337, The U.S.A.
2.	UV-VIS Spectrophotometer	APLE PD-303 UV, Japan
3.	Centrifuge	Hettich EBA 20, Germany
4.	Water bath	Techne junior TE-8J
5.	Deep freeze	ARCTIC 4612033, European Union, Denmark.
6.	Isotemp incubator	Fisher Scientific, France,0782.
7.	Shaker	France, 18682.
8.	pelvic ultrasonography	CT-R7V3.02-FTW-141204-EN, Coreano

2.2. Chemicals and kits

The chemicals and kits used are summarized in table 2.1.2

Table 2. 2 Chemicals and kits

No.	Material	Company
1.	Histidine-Rich Glycoprotein (HRG) ELISA kit.	CSB-E13159h, China.
2.	Human Inhibin B ELISA kit.	Lane 99 Jinhua Road, Pudong District, Shanghai, China.
3.	Anti Mullerian Hormone (AMH) ELISA kit.	Ansh labs, 445 Medical Center Blvd, Webster, TX77598-4217, USA.
4.	Copper manual kit.	SAE, Egypt.
5.	Iron manual kit.	Human, Max-Planck-Ring 1.65205 Wiesbaden, Germany.
6.	Zinc manual kit.	SAE, Egypt.
7.	Magnesium manual kit.	Human, Max-Planck-Ring 21.65205Wiesbaden, Germany.

2.3. Patients and controls

This study included thirty-three infertile women; those women were enrolled from outpatient women's clinic in the period from October 2016 to April 2017. Their ages range from (15 to 44) years old. The control group consisted of 22 healthy fertile women subjects who were free from signs and symptoms of diseases, their ages were matched with the age of infertile women.

The practical part was carried out in AL-Hussein Teaching Hospital of Karbala; a questionnaire was designed to obtain the information on an infertile female. It contained the name, age, weight, height, smoking, type of treatment, family history of infertility , education level, regular or irregular of menses , types of infertility, causes of infertility which are (tubal factor, endometriosis, and unexplained), number of children, duration of infertility and abortion history.

2.4. Collection of samples

Five milliliters of venous blood was drawn from the infertile women and the control group in the second day of men's to measure HRG, Inhibin B, AMH, Cu, Fe, Zn, and Mg concentrations.

Disposable syringes and needles were used for the collection of blood samples. The samples blood were centrifuged at 3000 rpm for 15 minutes. A serum of blood samples was taken from its tubes and put to freeze at -70 ºC until the analysis.

2.5 Methods

2. 5.1 Determination of Human Histidine-Rich Glycoprotein concentration.

HRG was determined by enzyme-linked immune sorbent assay (ELISA) system, by using human HRG ELISA kit (Cusabio CSB, China).

• Principle of assay

This assay employed the quantitative sandwich enzyme immunoassay technique. Antibody specific to HRG had been pre-coated onto a microplate. Standards and samples which are pipetted into the wells and any HRG present were bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for HRG was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of HRG bound in the initial step. The color development stopped and the intensity of the color is measured.

• Reagents and materials provided

The materials provided and reagents were: Assay plate (12 x 8 coated micro-wells) 1(96 wells), standard(Freeze dried) 2 , biotin-antibody(100 x concentrate) 1 x 120 µl , HRP- avidin (100 x concentrate) 1 x 120 µl, biotin-antibody diluent (1 x 15 ml) , HRP- avidin diluents (1 x 15 ml), sample diluent which contain on the [phosphate buffer salin|+ potassium tetraidomercric 0.01%] (1 x 50 ml), wash buffer (25 x concentrate) 1 x 20 ml, TMB (Tetra methyl benzenadin) substrate (1 x 10 ml), stop solution which contain on sulpharic acid (1 x 10 ml), adhesive strip (for 96 wells) (4), and instruction manual (1) .

• Preparation of reagents

All reagents were brought to room temperature 25^{0}C before use for 30 minutes.

1. Graduated containers were used to prepare the reagent.

2. Wash buffer was taken and used when the crystals had formed in the concentrate, warmed up to room temperature and mixed gently until the crystals had completely dissolved. 15 ml of wash buffer concentrate (20 xs) were diluted to preparation 300 ml of wash buffer (1 x).

3. A biotin-antibody (1x) vial has centrifuged before opening, a biotin-antibody was required a 100-fold dilution. A 100-fold dilution was suggested in 10 µl of biotin-antibody + 990 µl of biotin-antibody diluent.

4. An HRP-avidin (1x) vial was centrifuged before opening, an HRP-avidin has required a 100-fold dilution. A 100-fold dilution was suggested in 10 µl of HRP-avidin + 990 µl of HRP-avidin diluent.

5. The standard vial was centrifuged at 6000-10000 rpm for 30s, the standard was reconstituted with 1.0 ml of sample diluents, and the substitute did not diluent. The standard was varied to make sure complete reconstitution and permissible the standard to sit for a minimum of 15 minutes with gentle agitation prior to making diluents.

6. A 250 µl of sample diluent was pipetted into each tube (S0-S8), and the stock solution was used to produce a 2-fold dilution series (below), each tube was mixed thoroughly before the next transfer.

Tube	**S7**	**S6**	**S5**	**S4**	**S3**	**S2**	**S1**	**S0**
µg/ml	**400**	**200**	**100**	**50**	**25**	**12.5**	**6.25**	**0**

7. The diluent standard was served as the high standard (400µg/ml).

8. A sample diluent was served as the zero standards (0 µg/ml).

• Procedure

1-All reagents were ready, working standards and samples were equipped as directed in the previous sections.

2- The assay layout sheet was referred in order to determine the number of wells to be used any remaining wells and the desiccant back were put into the pouch and sealed the Ziploc, unused wells were stored at 4 °C.

3- A100 µl of standard and sample was added per well, and covered with the adhesive strip then incubated for 2 hours at 37°C. A plate layout was provided to record standard and samples assayed.

4- The liquid of each well was removed and not washed.

5- A100 µl of biotin – antibody (1x) was added to each well and covered with a new adhesive strip then incubated for 1 hour at 37°C.

6- The aspiration/wash process were repeated for two times for a total of three washes. Each well was washed by filling with wash buffer (200 µl) using a squirt bottle, multi-channel pipette, manifold disperser, or auto washer, then it was standing and let it for 2 minutes. The completed removal of liquid at each step was essential to good performance. After the last wash, any remaining wash buffer was removed by aspirating or decanting invert the plate and blot it against clean paper towels.

7- A100 µl of HRP-avidin was added to each well, which was covered with a new adhesive strip, and then it was incubated for 1 hour at 37 °C.

8-The aspiration/wash process was repeated for five times as in step 6. 9- A 90 µl of TMB substrate was added to each well, and then it was incubated for 15-30 minutes at 37°C.

10- A 50 µl of stop solution was added to each well, and gently the plate was tap to ensure thorough mixing.

11-The Optical Density (OD) of each well was determined within 5 minutes by using a microplate reader set to 450nm.

• Calculations

The concentration of HRG was calculated automatically by taken the average of each standard, sample and subtracted the average zero standard optical density. A standard curve was created by reducing the data by using computer software capable of generating a four parameter logistic (4-PL) curve-fit automatically. As an alternative, a standard curve was constructed by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and the best fit curve was drawn through the point on the graph.

2.5.2 Determination of Human Inhibin B concentration.

INH- B was determined by (ELISA) system, by using (INH- B) ELISA kit (Shanghai, China).

• Principle of assay

This assay uses (ELISA) based on biotin double antibody sandwich technology to assay (INH- B) is added to wells that are pre-coated with (INH- B) monoclonal antibody and then incubated. After incubation, anti INH-B antibodies were added and labeled with biotin to unite with streptavidin -HRP, which forms the immune complex. The unbound enzyme is removed after incubation and washing, the substrate A and B are added. The solution would turn blue and change to yellow with the effect of acid. The shades of the solution and the concentration of (INH-B) are positively correlated.

• Reagents and materials provided

The materials provided and reagents were: Coated ELISA plate (12-well* 8 tubes) , standard solution (1280 pg / ml) o.5ml x 1 , streptavidin-HRP (6 ml x 1) , stop solution (6 ml x 1), chromogenic reagent A (6 ml x

1) , chromogenic reagent B (6 ml x 1) , anti (INH-B antibodies labeled with biotin (1ml x 1) , standard dilution (3 ml x 1) , washing concentrate [(20 ml x 30) x 1] and instruction manual.

• Preparation of reagents

1-All reagents were brought to room temperature before use for 30 minutes.

2-Preparation of washing solution: The washing of concentration (30X) was diluted with distilled water for later use.

3- Dilution of standard solutions. The kit of inhibin B was providing one standard original concentration. The standard solution was diluted in small tubes as the following chart below:

640 pg/ml	**Standard No. 5**	**120 µl Original Standard + 120 µl Standard diluents**
320 pg/ml	**Standard No. 4**	**120 µl Original Standard No5 + 120 µl Standard diluents**
160 pg/ml	**Standard No. 3**	**120 µl Original Standard No 4 + 120 µl Standard diluents**
80 pg/ml	**Standard No. 2**	**120 µl Original Standard No 3 + 120 µl Standard diluents**
40 pg/ml	**Standard No. 1**	**120 µl Original Standard No 2 + 120 µl Standard diluents**

4-The number of stripes needed was determined by samples to be tested added to the standards. It was recommended that each standard solution and each blank would be arranged with multiple wells as much as possible.

• Assay Procedure

A-Sample injection

1- Blank well: Sample wasn't added, an anti INH-B antibody was labeled with biotin and streptavidin - HRP, chromogen reagent A and B and stop solution were added, each other step operation was the same.

2- Standard solution well: A 50 µl of standard and 50 µl of streptomycin-HRP (biotin antibodies have united in advance in the standard so no biotin antibodies are added) were added.

3- Sample well to be tested: A 40µl of the sample, 10µl of INH-B antibodies, and 50 µl of streptavidin- HRP were added. They covered it with seal plate membrane. It was shacked gently to mix then incubated at 37^0 C for 60 minutes.

B-Washing: Carefully the seal of plate membrane was removed, then it was drained liquid and it was shaken off the remainder. Each well was filled with a washing solution and its let stand for 30 seconds, and then it was drained. This procedure was repeated five times then it was blotted from the plate.

C- Color development: First 50 µl of chromogenic reagent A was added to each well, and then a 50 µl of chromogenic reagent B was added to each well. Gently, it was shacked to mix and was incubated for 10 minutes at 37ºC away from light for color development.

D-Stop: A 50 µl of stop solution was added to each well to stop the reaction (color changes from blue to yellow immediately at that moment).

E- Assay: A blank well was taken as zero and was measured the absorbance Optical Density (OD) of each well, one by one under 450 nm wavelength, which should be conducted within 10 minutes after having added stop solution.

F- According to standards concentrations and corresponding OD values, the linear regression equation of the standard curve was calculated.

Then according to the (OD) value of the sample, the concentration of the corresponding sample was calculated automatically.

● Calculation

On the graph paper, the standard curve was drawn. According to the OD value of the sample, which is the concentration of the sample was located.

2.5.3 Determination of Anti-Mullerian Hormone[85]

AMH was determined by (ELISA) system, by using human AMH ELISA kit (AnshLabs, USA).

• The principle of the assay.

This assay employed the quantitative three-step sandwich-type enzyme immunoassay technique. In the first step calibrators, controls and unknown samples are added to AMH antibody coated microtiter wells and incubated. After the second incubation and washing, the wells are incubated with streptavidin-horseradish peroxidase conjugate (SHRP) solution. After the third incubation and washing step, the wells are incubated with substrate solution (TMB) followed by an acidic stopping solution. In principle, the antibody -biotin conjugate binds to the solid phase antibody-antigen complex which in turn binds to the streptavidin-enzyme conjugate. The antibody-antigen - biotin conjugate - SHRP complex bound to the well is detected by the enzyme-substrate reaction. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as references filter.

The absorbance measured is directly proportional to the concentration of AMH / MIS in the samples and calibration.

• Reagents and materials provided

The materials provided and reagents were:

1- AMH/MIS Calibration A/Sample Diluent

One bottle.1ml labeled AMH/MIS Cal. A/Sample Diluent, containing 0 ng/ ml AMH in the protein-based buffer and pro-Clean 400. Store unopened at 2-8°C until the expiration date.

2- AMH/MIS Calibration B thru F (Lyophilized)

Five vials, labeled B-F, containing a concentration of approximately 0.09-15.0 ng/ml AMH in the protein-based buffer and Pro-Clean 400. Refer to calibration card for exact concentrations. Store unopened at 2 to 8°C until the expiration date reconstitute calibration B-F with 1ml of deionized water.

3-AMH/MIS Controls I and II (Lyophilized)

Two vials, labeled levels I and II containing low and high AMH concentrations in a protein-based buffer and Pro-Clean 400 refer to calibration card for exact concentrations. Store unopened at 2 to 8°C until the termination date. Reconstitute control levels I and II with 1 ml deionized water**.**

4- AMH/MIS Coated Micro titration strips.

One strip holder containing 12 strips and 96 microtitration wells with AMH antibody immobilized to the inside wall of each well.

5-AMH/MIS assay buffer.

One bottle of 12 ml containing a protein-based (BSA)-buffer with a non-mercury preservative.

6- AMH biotin conjugate ready to use (RTU).

One bottle 12 ml containing biotinylated anti-AMH antibody in protein-based buffer with a non-mercury preservative.

7- AMH/MIS Streptavidin-Enzyme conjugate ready to use (RTU).

One amber bottle 12 ml containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative.

8- TMB Chromogen solution.

One bottle of 12ml containing a solution of (TMB) in buffer with hydrogen peroxide.

9- Stopping solution.

One bottle of 12ml containing 0.2 M sulfuric acid.

10- Wash concentrate A.

One bottle 60ml containing buffered saline with a nonionic detergent and instruction manual.

• Preparation of reagents

All reagents were brought to room temperature 25 ^{0}C for 30 minutes before used.

1- AMH/MIS Calibration B-F and AMH/MIS Controls I and II: AMH/MIS Calibrate B-F and AMH/MIS Controls I and II each with 1ml deionized water. They were solubilized, then mixed well and used after they were reconstituted.

2- Wash Solution: Wash deliberate was diluted 25 - fold with deionized water. The wash solution is steady for one month at room temperature when stored in a tightly potted bottle.

3- Micro-titration Wells: The number of covered wells was chosen necessarily for the assay. The pouch with a desiccant.

• Assay Procedure.

1-AMH/MIS calibrator B-F and AMH/MIS controls I and Ⅱ were reconstituted with 1ml deionized water and solubilized for 10 minutes then were mixed well by gentle vortex.

2-The microtitration strips were labeled to be used.

3- A 25 µL of the (calibrator, controls, and unknowns) were pipetted to the appropriate wells.

4 - A100 µL of the AMH/ MIS assay buffer was added to each well using a repeater pipette.

5- The plate was incubated and shook at fast speed (600 - 800 rpm) on an orbital microplate shaker, for 90 minutes at room temperature 25^0C .

6- Each strip was aspirated and washed 5 times (350 µL/per well) with wash solution using an automatic microplate washer.

7-A100 µL of the antibody - biotin conjugate RTU was added to each well using a repeater pipette.

8-The plate was incubated and shook at fast speed (600 - 800 rpm) on an orbital microplate shaker, for 30 minutes at room temperature . The aspiration/wash process were repeated for 5 times as in step 6

9-A100 µL of the streptavidin-enzyme conjugate-RTU was added to each well using a repeater pipette.

10- The plate was incubated and shook at fast speed (600 – 800rpm) on an orbital microplate shaker, for 90 minutes at room temperature 25 ^{0}C.

11 - The aspiration/wash process were repeated 5 times as in step 6.

12- A100 µL of TMB chromogen solution was added to each well using a repeater pipette.

13 - The plate was incubated and sook at fast speed (600 - 800 rpm) on an orbital microplate shaker, for 8 -12 minutes at room temperature 25^0C .

14- A 100 µL of stopping solution was added to each well using a repeater pipette solution in the wells was read within 20 minutes, then a microplate reader set to 450 nm was used.

• Calculations

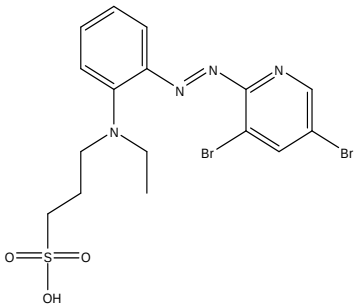
The concentration of AMH was calculated automatically by taking them to each standard, sample and subtracted the average zero standard optical density. A standard curve was created by reducing the data by using computer software capable of generating a four parameter logistic (4-PL) curve-fit automatically. As an alternative, a standard curve was constructed by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and the best fit curve was drawn through the point on the graph.

2.5.4 Determination of serum copper [86]

Copper was determined by the spectrophotometric method, by using a kit from LTA Company for biotechnology (Milano /Italia).

• Principle

The cupric ions are reacted with the chromogenic Di-Br-PAESA (3, 5-Dibromo-2-pyridylazo)-N-ethyl-N-(3-sulfopropyl) aniline forming a blue compound, whose intensity is proportional to the copper concentration present in the sample as in the following:

Cu^{+2} +  → Blue compound

Cupric ions chromogen Di-Br-PAESA

• Reagents

1. A standard (St.); contains 200 µg/dl of ion copper.
2. Reagent (A); contains 0.1 M of acetate buffer pH 4.9, reducing agents and preservatives.
3. Reagent (B); contains 0.1 M of 3,5 D-Br-pyridylazo)-N-ethyl-N-(3-sulfopropyl) aniline (3, 5 Di –Br- PAESA).

• Preparation of working reagent

Working reagent was prepared by mixing equal quantity of reagent A reagent B.

• Procedure

The steps of the procedure were followed in the following table:

Reagents	Blank	Standard	Sample
Work Reagent	1ml	1ml	1ml
Distilled water	66 µl	—	—
Standard	—	66 µl	—
Sample	—	—	66 µl

Each tube solution was mixed and waited for 10 minutes then the absorbance was read against the blank at 580 nm.

• Calculation

$$\text{Copper } (\mu g/dl) = \frac{\text{A (sample)}}{\text{A (standard)}} \times 200\ \mu g/dl$$

Where:

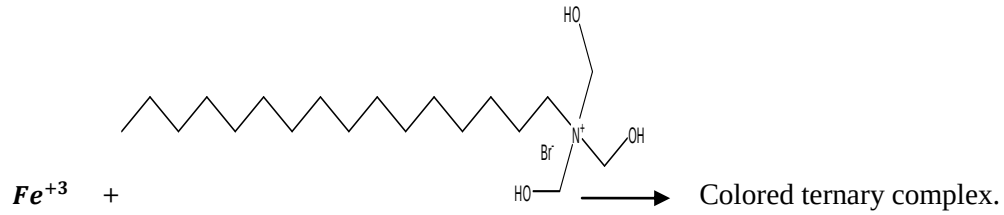
A = Absorbance

2.5.5 Determination of serum iron [87]

Fe was determined by the spectrophotometric method by using a kit from Germany Company for Human Gesellschaft for Biochemical.

• Principle

Iron (III) is reacted with the chromazurol B(CAB)and cetyl trimethyl ammonium - bromide (CTMA) to form a colored ternary complex with an absorbance of maximum at 623 nm as in the following:



Iron (III) CTMA

• Reagents

1. A standard (St.); contains 100µg/dl of ionized iron.
2. Reagents (R); contain 2.6 mol/L of guanidinium chloride, 45 mmol/l of sodium acetate buffer (pH 4.7) , 2.2 mmol/l of CTMA and 0.18 mmol/l of CAB.

• Procedure

The steps of the procedure were followed as the following table.

Pipette into cuvettes	Reagent blank(Rb)	Sample / Standard[STD]
Sample / [STD]	—	50 µl
Distilled water	50 µl	—
RGT	1000 µl	1000 µl

Each tube solution was mixed and incubated for 15 minutes at 20-25°C and the absorbance of the sample (A_{sample}) and the standard (A_{STD}) was measured against the reagent blank at 623 nm.

• Calculation

(Fe) concentration ($\mu g/dl$) = $\frac{A_{sample}}{A_{STD}} \times 100\ \mu g/dl$

Where:

A_{sample} =Absorbance of the sample.

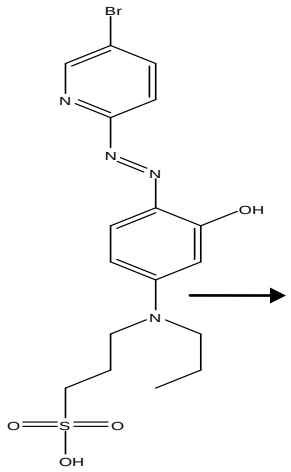
A_{STD} =Absorbance of the standard.

2.5.6 Determination of serum zinc[88]

Zinc was determined by the spectrophotometric method by using a kit from Egyptian company for biotechnology (S.A.E) /Egypt.

• Principle

Zn forms with 2-(5-Brom-2-pyridylazo)-5-(N-propyl-N sulfo propyl amino)-phenol (5-Br-PAPS) a red chelate complex. The increase of absorbance can be measured and is proportional to the concentration of total zinc in the sample as in the following:



Red chelate complex

Zn^{+2} +

Zinc 5-Br-PAPS

• Reagents

1. A standard (St.) solution; contains 200 µg/dl (30.6 of µmol / l).

2. Reagents (R); contain 0.02 mmol/L of 5-Br-PAPS, 200 mmol/L of bicarbonate buffer pH 9.8, 170 mmol/L of sodium citrate, 4 mmol/L of dimethylglyoxime and 1% of detergent.

• Procedure

The steps of the procedure were followed as the following table.

	Blank	Standard	Sample
Reagent	1ml	1ml	1ml
Standard	—	50 µl	—
Sample	—	—	50 µl

Each tube solution was mixed and incubated for 10 minutes at 37°C and the absorbance of the sample (A_{sample}) and the standard (A_{STD}) were measured against the reagent blank at 560 nm.

• Calculation

(Zn) concentration (µg/dl) = $\frac{\text{A specimen}}{\text{Standard}}$ × 100 µg/dl

Where:

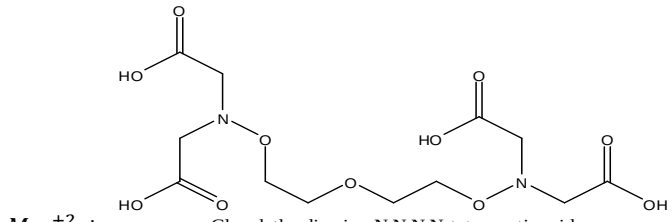
A= Absorbance.

2.2.7 Determination of serum magnesium [89]

Mg was determined by the spectrophotometric method by using a kit from Germany Company for Human Gesellschaft Biochemical.

• Principle

Magnesium ion in alkaline medium forms with Glycol etherdiamine - N, N, N, N-tetra acetic acid (GEDTA) to form a colored complex with xylyl blue. The increase of absorbance can be measured and is proportional to the concentration of total Magnesium in the sample as in the following:



Mg^{+2} + Glycoletherdiamine-N,N,N,N-tetra acetic acid ⟶ Coloured complex

Mg(II) GEDTA

• Reagents

1. A standard (St.) solution; contains 0.095% of sodium azide or 2.5 mg/dl of magnesium (II).

2. Reagents (R) ; contain 0.09 mmol/l of Xylidyl blue , 0.095% of sodium azide activators , 13 mmol/l of Glycol ether tetraacetic acid (GETA) and 50 mmol/l of 3-(Cyclohexylamino)-1-propane sulfonic acid (CAPS).

• Procedure

The steps of the procedure were followed the following table.

Pipette into cuvettes	Reagent blank	Sample or STD
Sample / STD	—	10 µl
Distilled water	10 µl	—
RGT	1000 µl	1000 µl

The steps were mixed and incubated for 10 minutes at 20-25°C and the absorbance of the sample (A_{sample}) and the standard (A_{STD}) were measured against the reagent blank at 520 nm.

• Calculation

(Mg) concentration (mg/dl) = $\frac{A_{sample}}{A_{STD}} \times$ 2.50 mg/dl .

Where:

A_{sample} = Absorbance of the sample.

A_{STD} =Absorbance of the standard.

2.8 Biostatistical analysis

The results are expressed as Mean ± Standard Error (SE). Students t-test and Pearson's correlation coefficients were used for analyzing results by using Statistical Package for the Social Sciences (SPSS) version 22.0. P-value ≤ 0.05 was considered significant [90].

Chapter three
Results
and
Discussion

3. Results and Discussion

3.1 The clinical characteristics of infertile women

The present study includes 33 infertile women and 22 control fertile women. The number of infertile women, age, BMI, family history, residency, education, and type of infertility, regulation of menses, abortion history, and causes of infertility were documented in (Table 3-1).

Table (3-1) The clinical characteristics feature of infertile women

Variables	No. = 33	%
Age(years)		
15 - 29	24	72.72
30 - 40	6	18.18
41 - 45	3	9.09
BMI(Kgm/m^2) :		
Normal (18.5-24.9)	12	36.36
Over weight (25-29.9)	14	42.42
Obese (> 35)	7	21.21
Family history :		
With family	10	30.30
Without family	23	69.69
Residency :		
City	24	72.72
Urban	9	27.27
Education :		
Uneducated	21	63.63
Educated	12	36.36
Type of infertility		
Primary	13	39.39
Secondary	20	60.60
Regulation of menses		
Regular	16	48.48
I rregular	17	51.51
Abortion:		
With abortion	10	30.30
Without abortion	23	69.69
Causes of infertility:		
Tubal factor	7	21.21
Endometriosis	4	12.12
Unexplained	22	66.6

3.2 Measurement of biochemical parameters in infertile women and control groups

3.2.1 A glycoprotein (HRG, Inhibin B, AMH)

Comparing the serum levels of glycoproteins HRG, inhibin B and AMH between infertile and healthy subjects: the results revealed that infertile women have a significantly lower serum inhibin B level than the healthy women ($p = 0.05$), whereas there were no significant differences in the concentration of the HRG and AMH levels ($p > 0.05$) (Table 3-2).

Table (3-2) The concentrations of HRG, Inhibin B and AMH of infertile and control groups

Parameters	Infertile women No.= 33 Mean ± SE	Control No.=22 Mean ± SE	P-value
HRG (ng/ml)	24.87 ± 7.17	48.46 ± 2.21	0.08
Inhibin B (pg/ml)	241.95 ± 20.43	319.34 ± 6.55	0.05
AMH (ng/ml)	5.30 ± 0.68	3.70 ± 0.48	0.06

Inhibin B does seem to be related to fertility, as low levels of inhibin B is associated with impaired ovulation, low pregnancy rates and increased danger of miscarriage [91]. Groome, *et al*. (1994) suggests that inhibin B a granulosa cell product plays a role in follicular growth with the possibility that serum inhibin B level correlates with follicular function and oocyte number [92].

Another study by Klein, *et al. (*1996) suggested that decreased inhibin B secretion was a reflection of a diminished ovarian follicular pool in older women. Magoffin and Jakimiuk, (1997) found that the amount of both inhibin proteins secreted into follicular fluid FF seems to increase with follicle development, although their concentrations may faintly decrease in the largest follicles due to strength in a greater fluid volume [93].

Inhibin B is an important indicator of ovarian reserve (the ovary's capacity to respond to gonadotropin stimulation) predicts the magnitude of retrievals, and is used to determine Ovarian Hyper-stimulation Syndrome (OHS) determines gonadotropin dosage for Assisted Reproductive Technologies (ART) [94]. Chang, *et al*. (2002) found that inhibin B in FF may serve as an effective marker for follicular development. They also showed a significant correlation between inhibin B levels in FF and embryo scores on the 2^{nd} or 3^{rd} day of their previous menstrual cycle, so they considered inhibin B a useful predictor of quality of the embryo [95] .

Histidine-rich glycoprotein interacts with other angiogenic factors, such as vascular endothelial growth factor VEGF and fibroblast growth factor FGF but no studies to date have shown how HRG affects angiogenesis in the follicle. Different isoforms of VEGF as well as various members of the FGF family, however, they have been shown to be involved in the regulation of folliculogenesis and oocyte development [96]. Apparently, the exact role of HRG in reproduction remains to be investigated as its exact bimolecular function is unclear [97]. HRG might be involved in several of the processes of importance for a pregnancy to occur but the exact role of HRG infertility is not yet fully described [60].

In this study, although there was no significant difference in the concentration of AMH, there was an increase in the level of AMH in infertile women group comparing with the control group. Recent studies have shown that AMH can be a good predictor of ovarian reserve and the success rates of *in vitro* fertilization IVF however; both AMH and FSH are still used as ovarian reserve test [98].

Serum levels of AMH and inhibin B could be good predictors to determine the number of immature oocytes retrieved IVF cycles [99]. More recently, some groups have reported the use of antral follicular count AFC and AMH in predicting the ovarian reserve. They conclude that AMH is a novel

marker of ovarian reserve [95]. Due to this stability, AMH was used as a marker for ovarian response to control ovarian stimulation independently of the day of the cycle in which the blood sample was obtained. Takahashi, *et al.* (2008) showed that there was no significant correlation between AMH levels and oocyte number [100].

3.2.2 Trace elements Cu, Fe, Zn and Mg

The trace elements which include (Cu, Fe, Zn, and Mg) were measured. The results showed a significant ($p = 0.004$) decrease in concentration of Zn and a highly significant ($p < 0.000$) increase in concentration of Mg in sera of infertile women compared with the control group whereas no significant ($p > 0.05$) differences in the concentration of Fe and Cu elements (Table 3.3).

Table (3.3) The concentrations of trace element (Cu, Fe, Zn, and Mg) of infertile women and control group

Parameters	Infertile women No. = 33 Mean ± SE	Control No. = 22 Mean ± SE	P-value
Cu (µg /dl)	138.48 ± 9.77	121.40 ± 5.70	0.13
Fe (µg /dl)	74.51 ± 9.29	69.00 ± 5.58	0.61
Zn (µg /dl)	82.93 ± 4.84	107.18 ± 6.84	0.004
Mg (mg/dl)	2.93 ± 0.15	2.10 ± 0.09	0.000
Cu/Zn	2.86 ± 0.77	1.23 ± 0.17	0.09

In the present study, zinc was in a low level in infertile women. The low level of zinc concentration may complicate such as impaired synthesis /secretion of FSH and LH, abnormal ovarian development, estrous cycle disruption and frequent abortion [101].

Jameson (1976) found that the low level of serum zinc and follicular fluid concentrations be related to increased infertility rate, also zinc is likely to be significant for reproduction [102].

In this study, there was a significant increase in the level of Mg in infertile women group comparing with control group. Studies that have evaluated magnesium levels in different settings might have to be re-appraised using a theoretical approach Liebscher and Liebscher have provided an example of re-appraisal [103], by increasing their intake of magnesium before undergoing fertility treatment, such as IVF. These infertile women can increase the chances of a successful conception and healthy pregnancy. While it is still unclear why low levels of magnesium affect a woman's fertility. There has been enough proof found for researchers to believe that there is a direct link between magnesium levels and fertility [104]. Mg is the most abundant cation in cells and the most abundant free divalent cation that is deeply and intrinsically involved in cellular metabolic processes. Mg-dependent enzymes appear in most metabolic pathways: for example, in many cases, there is a specific binding of Mg to biological membranes [105]. Plasma Mg was associated with hemoglobin. Both were inversely related to post-calving infection and uterine involution [106].

Copper imbalance is associated with every female organ related difficulty such as premenstrual syndrome, ovarian cysts, uterine fibroid tumors, infertility, miscarriages, and other menstrual problems. Women tend to have higher levels of copper than men. Women also have more symptoms related to copper imbalance [107].

A recent study found a link between low iron intake and female infertility, making increasing iron intake through food and iron supplements important for women trying to get pregnant, the lower the women's risk of developing ovulatory fertility problems. For example, women who consumed 41 milligrams or more of iron daily had a decreased risk of 62% of infertility problems. In addition, those women who were prescribed haem iron (iron that comes from animal sources, such as eggs) were also at higher risk for developing ovulatory fertility than those whose iron intake came from mostly

non-heme iron (non-animal sources of iron, such as beans) [108]. Many studies have shown that iron may be involved in ovulatory function and fertility. These studies have reported that the most prevalent nutritional deficiency worldwide is iron deficiency, therefore, an inverse association was observed between ovulatory infertility and both total iron and non-heme iron intake. Although, haem iron intake was accompanied by a greater risk of ovulatory infertility [109].

3.3 Correlation between the parameter under the study of infertile women

In this study, Pearson's correlation coefficient was used to mean the correlation between parameters in infertile women. The results revealed a medium positive significant correlation between Cu and Mg ($P<0.000$, $r=0.48$) whereas there was no significant correlation ($p>0.05$) between the other parameters (Table3-4). Also, the results showed that there is a linear relationship between Cu and Mg (Fig. 3.1).

Table (3-4) Correlation between the parameter under study of infertile women

Parameters	Parameters	R	P-Value
HRG (ng/ml)	Inhibin B (pg/ml)	0.13	0.33
	AMH (ng/ml)	- 0.12	0.37
	Cu (µg /dl)	- 0.14	0.30
	Fe (µg /dl)	0.09	0.50
	Zn (µg /dl)	0.06	0.64
	Mg (mg /dl)	-0.4	0.07
Inhibin B (pg/ml)	AMH (ng/ml)	- 0.03	0.82
	Cu (µg /dl)	0. 03	0.79
	Fe (µg /dl)	- 0.17	0.20
	Zn (µg /dl)	- 0.10	0.45
	Mg (mg /dl)	0.07	0.60
AMH (ng/ml)	Cu (µg /dl)	0.02	0 .83
	Fe (µg /dl)	0.05	0.66
	Zn (µg /dl)	- 0.17	0.21
	Mg (µg /dl)	0.13	0.34
Cu (µg /dl)	Fe (µg /dl)	0.13	0.33
	Zn (µg /dl)	- 0.21	0.12
	Mg (mg /dl)	0.48*	0.000
Fe (µg /dl)	Zn (µg /dl)	- 0.15	0.27
	Mg (mg /dl)	0.05	0.71
Zn (µg /dl)	Mg (mg /dl)	-0.10	0.44

*** Correlation is significant at the 0.01 level (2-tailed)**

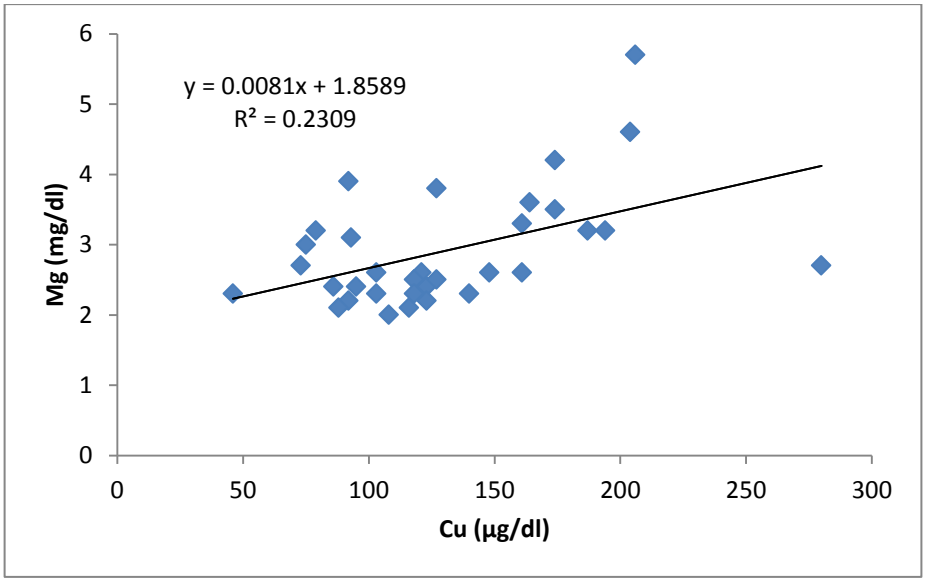


Fig. (3.1) Correlation between Cu and Mg of infertile women

Nutrients such as copper and magnesium play a vital role in the treatment of infertility with proper physical activity [110].

Magnesium can even help women with fertility problems because it relieves fallopian tube spasm that can prevent egg implantation. Therefore, the requirement for magnesium begins from the first day of conception and continues through life [111].

Deficiencies of trace elements like copper and magnesium have been occupied in various reproductive events like infertility; trace element copper is involved in the role of several cuproenzymes that are essential for life, magnesium a crucial trace element for the human body that is needed for the proper bone formation and in various intracellular enzymatic processes. Magnesium has established its role in obstetrics [112].

3.4 Type of infertility

Infertile women group was classified into two subgroups according to the type of infertility, these groups were primary and secondary infertility.

3.4.1 Measurement of glycoproteins (HRG, inhibin B, AMH)

The present study did not show any significant ($p > 0.05$) differences in the concentration of HRG, inhibin B and AMH between the two types of infertility in infertile women group (Table 3-5).

Table (3- 5) The concentrations of (HRG, inhibin B, and AMH) of primary, secondary infertile women

Parameters	Primary infertility No.= 13 Mean ± SE	Secondary infertility No. = 20 Mean ± SE	P-value
HRG (ng/ml)	22.83 ± 8.60	26.19 ± 10.60	0.80
Inhibin B (pg/ml)	217.63 ± 33.81	257.76 ± 25.65	0.35
AMH (ng/ml)	5.77 ± 1.27	5.00 ± 0.79	0.59

According to a study published at the end of 2012 by the World Health Organization (WHO), infertility is distributed into two groups, primary and secondary [113].

Primary infertility defined as the inability to achieve a pregnancy after 12 months of unprotected intercourse and secondary infertility defined as the inability to conceive after previously experiencing a successful pregnancy [114]. Infertility, whether primary or secondary, though affects couples, it is an experience that strikes at the very core of a woman's life [115].

Secondary infertility has the same causes as primary infertility when a pregnancy has never occurred despite regular, unprotected intercourse [116].

The women with secondary infertility are socially stigmatized and have to bear the brunt of being infertile, irrespective of who is responsible for infertility. Daiter (2007) who found that hormonal causes for recurrent pregnancy loss are generally considered luteal phase defects. Luteal phase defect is most often resulting from inadequate progesterone which affects the endometrial lining [117]. The study of Newton, *et al*. (1999), Epstein and Rosenberg, (2005); McQuillan, *et al*. (2007) found no significant differences in concentrations levels of distress and infertility related stress for women with primary and secondary infertility[114].

3.4.2 Measurement of trace elements (Cu, Fe, Zn, and Mg)

The present study did not show any significant ($p > 0.05$) differences in the concentration of Cu, Fe, Zn and Mg between the two types of infertility in infertile women group (Table 3-6).

Table (3-6) The concentrations of trace elements (Cu, Fe, Zn, and Mg) of primary and secondary infertile women

Parameters	Primary infertility No.= 13 Mean ± SE	Secondary infertility No. = 20 Mean ± SE	P-value
Cu (µg /dl)	134.38 ± 11.47	141.15 ± 14.51	0.71
Fe (µg /dl)	60.76 ± 15.50	83.45 ± 11.42	0.23
Zn (µg /dl)	86.92 ± 6.38	80.35 ± 6.89	0.49
Mg (Mg/dl)	3.08 ± 0.28	2.84 ± 0.16	0.43
Cu/Zn	1.66 ± 0.19	3.63 ± 1.24	0.21

The results of the current study were disagreement with Soltan and Jenkins(1983) who found that plasma copper concentration was significantly lower in infertile women than in control subjects and somewhat lower in women with secondary rather than primary. Plasma zinc concentration was not appreciably different in infertile and fertile women. Low plasma copper concentration may influence normal human female fertility [118].

Plant-based iron foods and iron supplements were also shown to decrease infertility. (It's important to note that the same findings don't apply to iron from red meat). This Nurse's Health study showed that women who took plant-based iron supplements have a 40 % reduction in infertility related to ovulation. Iron is needed to make estrogen and progesterone, essential for normal ovulation. In a study on unexplained infertility, it was found that those women with iron levels (measured by serum ferritin) below 40 µg/dl had a harder time conceiving [119]. The combined effect of Fe deposition and increased OS (because of a significant prooxidants/antioxidants imbalance results in the dysfunction of the female. Also, the Fe mediated effect on fertility could be discriminated in its hypothalamic-pituitary effect and in its effect on the female reproductive system [78].

Iron is essential for women to have good levels of iron while trying to conceive during pregnancy. Symptoms of iron deficiency include fatigue, breathlessness, dizziness and unusual pallor and if you experience these or have previously had low iron levels [120].

In early life, zinc deficiency may affect embryogenesis and may influence the duration of pregnancy. Additional exacerbating factors include high physiological requirements, excessive losses by pathological conditions, intestinal failure and treatment with some drugs. Zinc is a crucial element of the immune response. The presence of zinc is essential to ensure the normal activity of enzymes, peptides, and cytokines in the cells of the immune system [121].

Magnesium is a necessary cofactor utilized by 700-800 enzyme systems that perform vital metabolic functions in the body [122]. Several studies in developed countries have reported reduced rate of preterm delivery and intrauterine growth restriction among peoples given supplements of magnesium [123].

3.4.3 Correlation between parameters under study in primary infertile women

In this study, Pearson's correlation coefficient was used to determine the correlation between parameters in primary infertile women group. The results revealed a medium positive significant correlation between inhibin B and Mg (P=0.03, r=0.58) and a negative correlation between Cu and Fe (p= 0.05, r =-0.54) whereas no significant correlation between the other parameters under study (Table 3.7). Also, the results showed that there is a linear relationship between inhibin B and Mg and a negative correlation between Cu and Fe (Fig. 3.2) and (Fig. 3.3) respectively.

Table (3-7) Correlation between parameters under study HRG, Inhibin B, and AMH and trace elements tests (Cu, Fe, Zn, and Mg) in primary infertile women

Parameters	Parameters	R	P – value
HRG (ng/ml)	Inhibin B (pg/ml)	0.34	0.24
	AMH (ng/ml)	- 0.42	0.14
	Cu (µg /dl)	-0.26	0.37
	Fe (µg /dl)	0.42	0.14
	Zn (µg /dl)	0.32	0.27
	Mg (mg /dl)	0.18	0.55
Inhibin B (pg/ml)	AMH (ng /ml)	0.0	0.96
	Cu (µg /dl)	-0..0	0.75
	Fe (µg /dl)	- 0.37	0.21
	Zn (µg /dl)	0.24	0.41
	Mg (mg /dl)	0.58*	0.03
AMH (ng/ml)	Cu (µg /dl)	- 0.16	0.59
	Fe (µg /dl)	0.14	0.64
	Zn (µg /dl)	0.18	0.54
	Mg (mg /dl)	0.47	0.10
Cu (µg /dl)	Fe (µg /dl)	-0.54**	0.05
	Zn (µg /dl)	0.16	0.58
	Mg (µg /dl)	- 0.002	0.99
Fe (µg /dl)	Zn (µg /dl)	0.25	0.39
	Mg (mg /dl)	0.33	0.26
Zn (µg /dl)	Mg (mg /dl)	- 0.05	0.84

*Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.05 level (2-tailed)

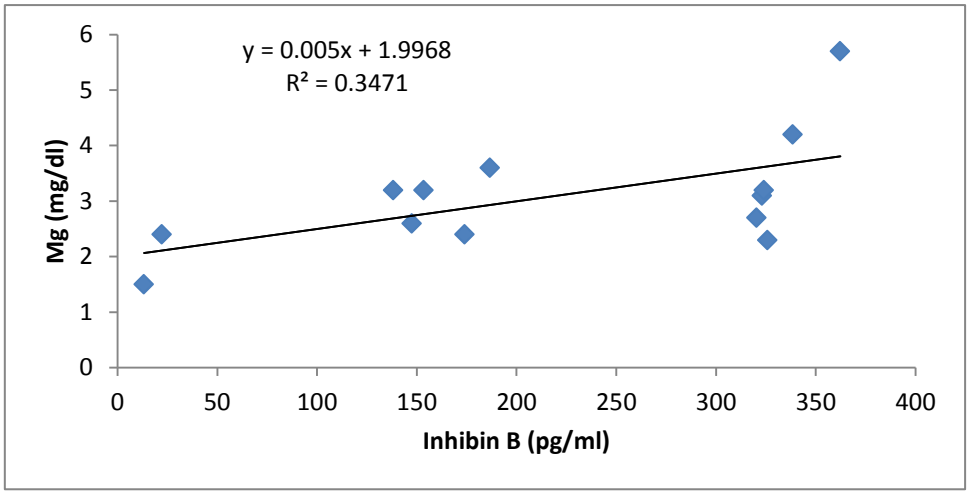


Fig. (3.2) Correlation between inhibin B and Mg in primary infertile women

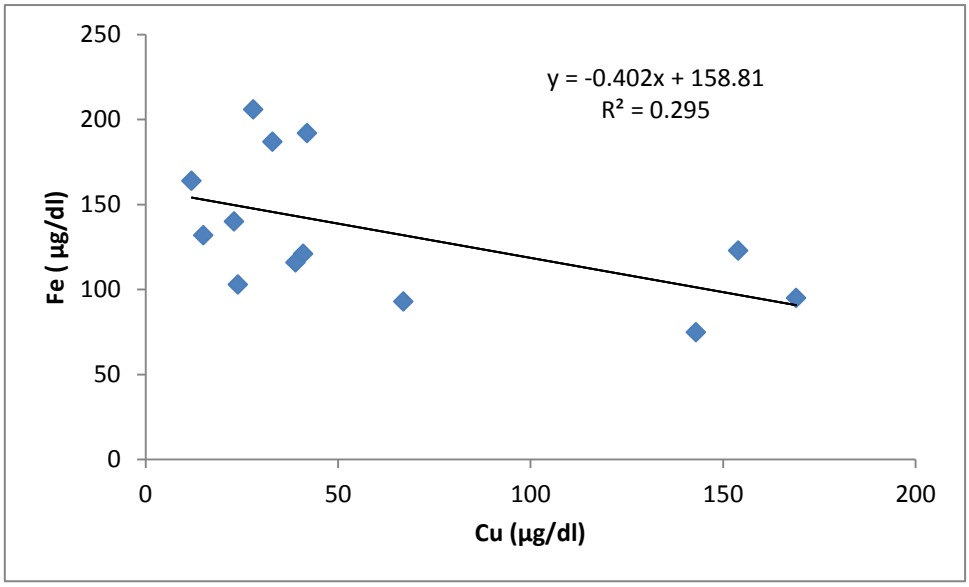


Fig. (3.3) Correlation between Fe and Cu in primary infertile women

In this study, there was a medium positive significant difference in the concentration of inhibin B and Mg. In addition, the result showed a negative significant difference in the concentration of Cu and Fe. So there were no studies about the correlation between (inhibin B, Mg) and (Cu, Fe) in primary women infertility [a novel result].

3.4.4 Correlation between parameters under study in secondary infertile women

In the present study, by using Pearson's correlation coefficient, the result showed a significant negative correlation (r=-0.43, p=0.05) between HRG and Mg in the secondary infertile women group whereas no significant correlation between the other parameters under study (Table 3.8). Also, the results showed that there is a linear relationship between HRG and Mg (Fig. 3.4).

Table (3-8) Correlation between parameters under study in secondary infertile women

Parameters	Parameters	r	P – value
HRG (ng/ml)	Inhibin B (pg/ml)	0.003	0.99
	AMH (ng/ml)	- 0.14	0.54
	Cu (µg /dl)	- 0.09	0.69
	Fe (µg /dl)	0.13	0.58
	Zn (µg /dl)	- 0.30	0.19
	Mg (mg /dl)	- 0.43*	0.05
Inhibin B (pg/ml)	AMH (ng/ml)	- 0.03	0.87
	Cu (µg /dl)	0.06	0.79
	Fe (µg /dl)	- 0.40	0.07
	Zn (µg /dl)	0.09	0.68
	Mg (µg /dl)	- 0.28	0.21
AMH (ng/ml)	Cu (µg /dl)	0.34	0.13
	Fe (µg /dl)	- 0.06	0.75
	Zn (µg /dl)	0.15	0.52
	Mg (µg /dl)	0.02	0.91
Cu (µg /dl)	Fe (µg /dl)	- 0.27	0.24
	Zn (µg /dl)	0.03	0.87
	Mg (µg /dl)	0.01	0.95
Fe (µg /dl)	Zn (µg /dl)	- 0.05	0.80
	Mg (µg /dl)	- 0.21	0.36
Zn (µg /dl)	Mg (µg /dl)	0.09	0.68

*Correlation is significant at the 0.05 level (2-tailed)

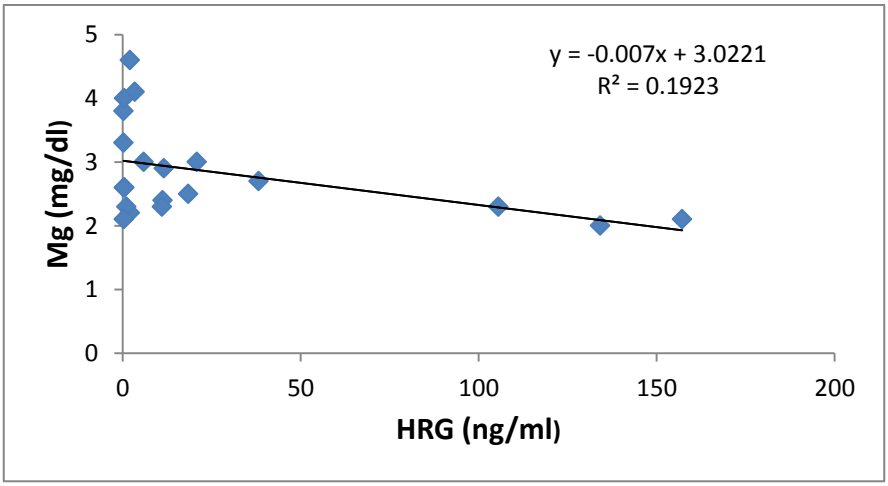


Fig. (3.4) Correlation between HRG and Mg in secondary infertile women

In this study, there was a negative significant difference in the concentration of HRG and Mg to date. No study examine the correlation between (HRG and Mg) in secondary women infertility [a novel result].

3.5 Follow up of infertile women

The treatment group of 33 infertile women was followed up for four months after the treatment with medication drug (Gonadotropin and Letrozole), and then the parameters under study were measured.

3.5.1 Measurement of glycoprotein (HRG, Inhibin B, AMH)

The result showed a significant decrease in AMH concentration ($p = 0.04$) and a significant increase in HRG concentration ($p= 0.01$) among infertile women whereas there was no significant differences change in inhibin B level ($p > 0.05$ (Table 3-9).

Table (3-9) The concentrations of (HRG, Inhibin B, and AMH) of infertile women before and after treatment

Parameters	Before treatment No. = 33 Mean ± SE	After treatment No. = 33 Mean ± SE	P-value
HRG (ng/ml)	24.89 ± 7.17	57.96 ± 10.77	0.01
Inhibin B(pg/ml)	242.00 ± 20.43	283.77± 19.70	0.14
AMH (ng/ml)	5.55 ± 0.65	3.87 ± 0.49	0.04

Gonadotropin therapy plays an integral role in ovarian stimulation for infertility treatments. Efforts have been made over the last century to improve gonadotropin preparations. Undoubtedly, current gonadotropins have better quality and safety profiles as well as clinical efficacy than earlier ones. A major achievement has been introduced recombinant technology in the manufacturing processes for follicle-stimulating hormone, luteinizing hormone, and human chorionic gonadotropin [124].

Gonadotropins play a significant role in the secretion of several substances by granulosa cells (eg: hyaluronic acid) in turn affecting oocyte development and maturation [125] as well as treatment of infertility. It is highly specific and depends on the reason. Anovulatory infertility and polycystic ovary syndrome are commonly treated with clomiphene and occasionally with metformin or tamoxifen Gonadotrophins (follicle stimulating hormones) that may be used in women whose clomiphene has been unsuccessful [129]. Gonadotrophin therapy is based on the physiological concept that initiation and maintenance of follicle growth may be achieved by a transient rise in FSH above a threshold dose for sufficient duration to generate a limited number of developing follicle [130].

Studying of HRG genotype affects the number of fertilized oocytes. Women who were given lower gonadotropins dosages obtained the most fertilized oocytes. The number percentage did not differ based on HRG genotype. This implies that the HRG does not appear to affect oocyte maturity [131].

The level of AMH slowly drops after gonadotropin treatment during controlled ovarian stimulation. This decrease could be an effect of a direct or indirect negative influence of FSH on AMH ovarian secretion. The exogenous FSH treatment elevates estradiol, which could be the source of AMH decrease as estradiol negatively influences the regulation of AMH in the ovary. It is recommended to use low initial doses of gonadotropins and protocols with a GnRH antagonist. It is also important to direct women to fertility treatment facilities as soon as possible to select the appropriate treatment [9].

AMH has also been suggested to apply a physiological effect on antral follicles in the human ovary before final selection. There exists a fine-tuned and delicate balance between estradiol E2 and inhibin output by the preovulatory follicle and gonadotropin secretion by the pituitary to make sure that ovulation is triggered exactly at the right time. Recently, it has been suggested that AMH may exert a physiological role in down-regulating the aromatizing capacity of granulosa cells until the time of follicular selection [132].

In this study, the results disagree with other studies when they reveal increased serum inhibin B levels in infertile women during gonadotropin treatment, attributed to production by follicles. Early follicular phase antral follicle number positively correlates with total and mature oocyte numbers after gonadotrophins stimulation for IVF and is linked to inhibin B, androgen, and insulin in predicting ovarian follicle recruitment by gonadotropins [133].

Letrozole has become an important tool in our armamentarium for treating infertility, yet surprisingly little effort has been devoted toward optimizing its effectiveness [134]. The use of aromatase inhibitors such as letrozole in the initial follicular phase has a negative feedback effect on the hypothalamus and pituitary glands thereby causing GnRH, LH and FSH secretion with resultant ovarian follicular growth stimulation. It seems that letrozole and its drug group are safe, reliable and cheap drugs with therapeutic value. It is probable that letrozole does not produce deleterious effects similar to that found with clomiphene citrate on the endometrium, although it can lead to pregnancy [135].

3.5.2: Measurement of trace elements (Cu, Fe, Zn, and Mg)

The trace elements which include (Cu, Fe, Zn, and Mg) were measured before and after treatment. The results showed a significant ($p < 0.000$, $p = 0.004$) decrease in concentration of Zn , Mg and Cu / Zn respectively in infertile women after treatment compared with the infertile women before treatment, whereas no significant ($p > 0.05$) differences in the concentration of sera Fe and Cu elements (Table 3.10).

Table (3.10) The concentrations of Cu, Fe.Zn, Mg) of infertile women before and after treatment

Parameters	Before treatment No. = 33 Mean ± SE	After treatment No. = 33 Mean ± SE	P-value
Cu (µg /dl)	135.36 ± 9.70	139.51 ± 8.50	0.74
Fe (µg /dl)	74.51± 9.29	96.63± 7.46	0.06
Zn (µg /dl)	82.93 ± 4.84	49.84 ± 4.25	0.000
Mg (Mg/dl)	2.93 ± 0.15	2.36 ± 0.11	0.004
Cu/Zn	2.17 ± 0.42	3.52 ± 0.46	0.03

Trace elements can act with all levels of production, action, the activity of peripheral receptors that allow the expression of hormonal message and regulation of hormones [136]. Zinc is a very important element in the reproductive cycle of species. In humans, it is necessary for the formation and maturation of spermatozoa, for ovulation, and for fertilization. During pregnancy, zinc deficiency causes a number of anomalies: spontaneous abortion, pregnancy-related toxemia, extended pregnancy or prematurity, malformations, and retarded growth. Delivery is adversely affected by the deficiency. These different effects of zinc can be explained by its multiple actions on the metabolism of androgen hormones, estrogen, and progesterone, together with the prostaglandins [137].

Recent findings show that zinc is an important factor necessary for regulating the meiotic cell cycle and ovulation. However, the role of zinc in promoting oocyte quality and developmental potential is not known [138].

Magnesium is well known in the world of obstetrics for many important uses. Many women in our modern society are magnesium deficient due to low dietary intake, and low dietary magnesium intake resulting in hypomagnesemia that has recently been shown to have many deleterious effects. Magnesium uses are wide-reaching, touching many areas of women's health and gynecology [81].

3.5.3: The effect of gonadotropin and letrozole on treatments and size of the Follicles

Pelvic ultrasonography technique was used to measure the follicle sizes after ovary stimulation in the treatment group. The results showed a significant (p=0.000) increase in the size of follicles to become mature oocytes in both left and right ovary to befor treatment case. (Table 3.10).

Table (3-11) The size and number of Follicles of infertile women before and after treatment

	Before treatment No. = 33 Mean ± SE	After treatment No. = 33 Mean ± SE	P-value
Size of Follicles	8.015 ± 0.21	20.03 ± 0.53	0.000
No. of Follicles	19.15 ± 1.11	25.4 ± 1.50	0.000

Durlinger, *et al*. (2002) demonstrated that AMH and inhibin B are produced by granulosa cells and appear to have both autocrine and paracrine effects within the follicle. The oocyte increases in size as the follicle develops more layers of granulosa cells in the pre-antral follicles. The later stages of follicle maturation from antrum formation to ovulation are gonadotrophin: dependent and are highly regulated by the cyclical changes in LH and FSH. During these gonadotrophin dependent stages, the primary oocyte does not grow significantly but undergoes both cytoplasmic and nuclear maturation [139].

During the luteal phase of letrozole-associated Controlled Ovarian Stimulation (COS) cycles (triggered with human chorionic gonadotrophin (hCG)) progesterone levels are similarly elevated to those obtained after standard COS without letrozole. Letrozole was shown to be effective in maintaining infra-physiological estrogen levels while harvesting several mature oocytes for subsequent oocyte or embryo vitrification [140].

3.6 The demographic study

3.6.1 Age factor

In this study, infertile women were classified into three groups according to their ages, group1 their ages are ranging from (15 to 29) years old, group 2 their ages are ranging from (30 to 40) years old and group 3 their ages are ranging from (41 to 45) years old, the percentage of these groups were 72.72 %, 18.18% and 9.09% respectively (Fig. 3.5).

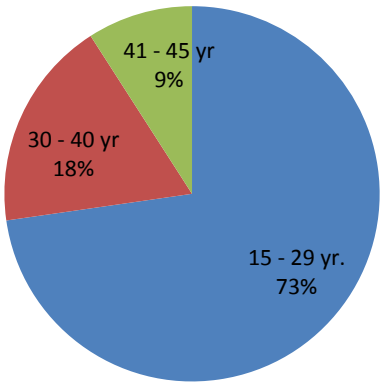


Fig. (3.5) The percentage of infertile women according to their ages

The infertile women were classified into two groups according to their ages, group1 their ages are from (15 to 29) years and group2 their ages are from (30 to 45) years. The results show significant ($p < 0.05$) decrease in concentration of Cu and Fe in infertile women which their ages are from (15 to 29) years, compared with infertile women their ages are from (30 to 45) years.

Whereas no significant differences in the concentration of parameters under study between two groups (Table 3.12). Also, the results showed that there is a linear relationship between ages and iron (Fig. 3.6).

Table (3-12): The concentrations of parameters under study in sera of infertile women with two groups according to age

Parameters	(15-30) yr. No. = 11 Mean ± SE	(31-45) yr. No. = 12 Mean ± SE	P-value
HRG (ng/ml)	58.72 ± 21.02	43.67 ± 15.32	0.60
Inhibin B (pg/ml)	337.91 ± 25.22	310.67 ±52.88	0.60
AMH (ng/ml)	4.56 ± 1.09	3.30 ± 0.49	0.09
Cu (µg /dl)	132.00 ± 22.94	147.33 ± 42.12	0.04
Fe (µg /dl)	53.57 ± 5.93	72.46 ± 9.22	0.001
Zn (µg /dl)	112.85 ± 7.45	104.53 ± 9.50	0.25
Mg (Mg/dl)	2.60 ± 0.40	3.01 ±0.41	0.06

In addition, by using Pearson's correlation coefficient, the results showed that there was a positive significant ($r= 0.71$, $p< 000$) correlation between Fe and age whereas no significant ($p>0.05$) differences in concentration of other parameters under study (Table 3.13). Also, the results showed that there is a linear relationship between ages and iron (Fig. 3.6).

Table (3.13) Correlation between parameters under study and age of infertile women

Parameters	r	P-value
HRG (ng/ml)	0.21	0.22
Inhibin B (pg/ml)	- 0.05	0.75
AMH (ng/ml)	- 0.25	0.14
Cu (µg /dl)	- 0. 23	0.18
Fe (µg /dl)	0.71*	0.000
Zn (µg /dl)	- 0.15	0.40
Mg (Mg/dl)	- 0.26	0.13

*Correlation is significant at the 0.01 level (2-tailed)

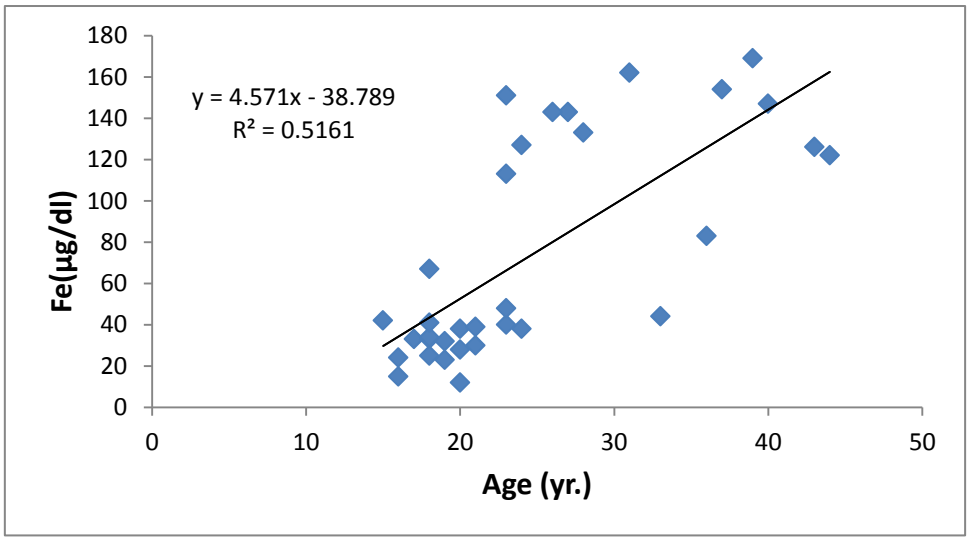


Fig. (3.6) the correlation between ages and iron of infertile women

Many factors, such as age, play a role in a pregnancy's success that can affect the quantity and quality of the oocytes found in the follicles [141]. According to the statistics on female age and declining fertility, there is a slow decline in pregnancy rates in the early 30%, while it becomes more substantial in the late 30% and early 40'%. In addition to the decline in pregnancy, miscarriage rates also increase as the mother ages and very few women over 44years old are still fertile [142].

Other studies showed that women who do not get sufficient amounts of iron may suffer from anovulation (lack of ovulation) and possibly poor egg health, which can inhibit pregnancy at a rate 60% higher than those with sufficient iron stores in their blood [143].

There are few studies investigating the effect of Fe overload (toxicity) and fertility, and even less with regard to female fertility. Excess Fe leads to reduced production of the hormones LH and FSH from the anterior pituitary, suggesting impaired oocyte maturation and low ovarian reserve and infertility, likewise in patients with hemochromatosis, a genetic condition that leads to increasing serum Fe levels and also subfertility in women [144].

The result of the current study is consistent with the study of Miller *et al.* (1999) who suggests that a diagnosis of ovulatory dysfunction was more common in younger age group. However, they could only show a trend towards an increased prevalence of unexplained infertility in older women [145].

Domingues *et al.* (2010) found decreased inhibin B corresponding with age makes inhibin B to be a marker of ovarian activity rather than ovarian reserve [146]. Subsequent studies demonstrated that inhibin B is the main suppressor of FSH in the early follicular phase. With increasing age, the falling number of follicles present results in lower inhibin B production and FSH consequently rises [147].

Earlier studies using immunoreactive inhibin measurements found that ovarian inhibin secretion decreases with declining ovarian function with advancing age as a result of a reduced cellular function rather than a decrease in the follicular population [148].

The result of current study disagree with result of Laven, *et al*. (2006) and Serdar Aydın, *et al.* (2015) who found correlation between age and serum AMH levels [145,149], due to concomitant decline in the number of primordial follicles, and intern decrease the number of granulosa cells and decreased inhibin B corresponding with age makes it to be a marker of ovarian activity rather than ovarian reserve [145].

3.6.2 Body Mass Index (BMI) factor

In the present study, infertile women were classified into three groups according to body mass index (BMI), the normal range of infertile women (18.5 - 24.9), overweight (25- 29.9) and obese (>35), the percentage of these groups were 36.36 %, 42.42% and 21.21% respectively (Fig. 3.7).

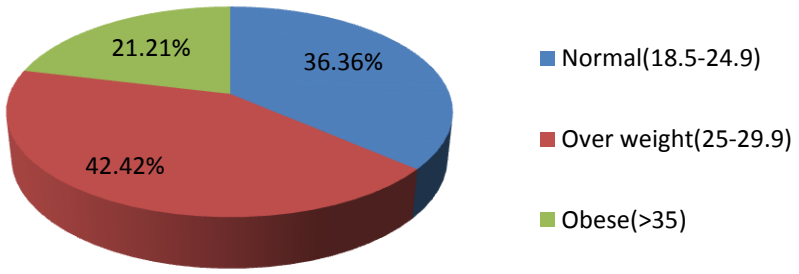


Fig. (3.7) The percentage of infertile women according to their BMI

The infertile women were classified into two groups depending on the BMI value, obese and non-obese women. The results did not show any significant ($p > 0.05$) differences in concentration of parameters under study between the two groups (Table 3.14).

Table (3.14) BMI for parameters under the study of obese and non –obese of infertile women

Parameters	Obese infertile No. = 7 Mean ± SE	Nonobese infertile No. = 26 Mean ± SE	p-value
HRG (ng/ml)	24.57± 12.46	24.97± 8.72	0.97
Inhibin B (pg/ml)	216.50 ± 58.40	250.09 ± 20.13	0.49
AMH (ng/ml)	5.14 ± 1.45	5.35 ± 0.79	0.67
Cu (µg /dl)	155.25 ± 29.98	133.12 ± 9.91	0.34
Fe (µg /dl)	87.87± 19.69	70.24 ± 10.61	0.44
Zn (µg /dl)	77.75 ± 7.17	84.60 ± 6.00	0.57
Mg (Mg/dl)	2.71 ± 0.19	3.00 ± 0.18	0.44
Cu/Zn	2.46 ± 9.44	2.98 ± 1.01	0.77

By using Pearson`s correlation coefficient, the results demonstrated no significant correlation between BMI and the parameters under study (Table 3.15).

Table (3.15) The correlation between BMI (kg/m²) and parameters understudy of infertile women

Parameters	r	P-value
HRG (ng/ml)	- 0.07	0.68
Inhibin B (pg/ml)	0.03	0.84
AMH (ng/ml)	- 0. 05	0.75
Cu (µg /dl)	0.05	0.78
Fe (µg /dl)	0.12	0.48
Zn (µg /dl)	- 0.04	0.80
Mg (Mg/dl)	- 0.20	0.26

According to the World Health Organization (WHO, 2000), lean is defined as a BMI < 18.5 kg/m^2, normal weight as BMI between 18.5 and 24.8 kg/m^2, overweight as BMI between 25.0 and 29.9 kg/m ^{2} and obese as BMI ≥ 30 kg/m ^{2} [54].

The possible complications with increased BMI in women trying to get pregnant include hypertension, gestational diabetes, pre-eclampsia, and stillbirth [113].

Obesity defined as BMI ≥ 30 kg/m^2 is increasing around the world [150]. Obesity is an increasingly prevalent health hazard and causes many disorders of female reproduction [151]. Obesity affects approximately half of the general population and is thus a common problem among the fertile population. Obese women have a higher prevalence of infertility compared with their lean counterparts [33].

The current study showed that there are no significant differences in serum levels of HRG, inhibin B and AMH between obese and non-obese women. There was no significant correlation between BMI and the serum of ovarian reserve [152].

In this study, the results disagree with another study by Biritwum, *et al*. (2006), who revealed that the prevalence of obesity was found to be higher among females [153].

Richard, (2007) reported that all the obese patients increased in serum prolactin level and decreased FSH levels. The hypothalamus, through the release of gonadotropin-releasing hormones, controls the pituitary gland which directly or indirectly controls most other hormonal glands in the human body. Thus, alterations in the chemical signals from the hypothalamus can affect the pituitary gland, ovaries, thyroid, mammary gland and hence, hormonal abnormalities. Hormonal abnormalities that affect ovulation include hyperthyroidism, hypothyroidism, polycystic ovary syndrome and hyperprolactinemia [33].

No study has evaluated the independent effect of obesity and hyperinsulinemia on the level of inhibin B in women without hyperandrogenism [151].

The concept that BMI may play a role in inhibin B regulation as inhibin B serves as a marker of small antral follicle growth. The suppression of inhibin B with increased BMI suggests that granulosa cell or follicular production of inhibin B and possibly follicle health decreased with obesity. This may be true even in regularly cycling obese women because obesity itself is associated with anovulation [154].

Obesity and overweight may be associated with poor dietary Fe intake. Studies that have estimated dietary Fe intake in women have not shown lower intake of this mineral in obese women. No significant differences in Fe intake were found by comparing BMIs and results were reported by Cepeda, *et al*. (2011) who did not show differences in Fe intake by BMI classification [155].

Zinc may be allied with the energy homeostasis of obesity via its interaction with dietary fat consumption, but zinc values show no changes during the oral glucose tolerance test in obese individuals [156].

3.6.3 Family history

The infertile women were classified into two groups: group1 with a family history of infertility (30.30 %) and group2 without a family history of infertility (69.69 %) (Fig. 3.8).

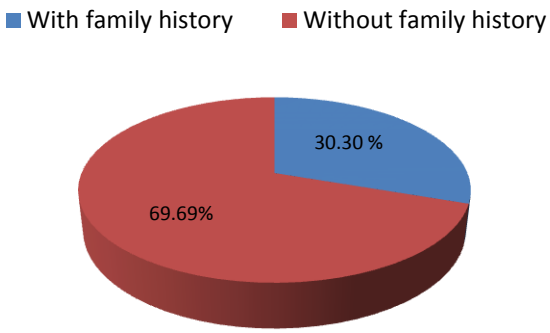


Fig. (3.8) The percentage of infertile women with and without a family history

The results showed a significant (p=0.03) decrease in concentration of HRG in the serum of infertile women with family history group compared with infertile women without family history group and significant (p=0.02) decrease in the concentration of Fe in the serum of infertile women with history family group compared with infertile women without family history group. In addition, the results revealed a significant increase in the concentration of Mg (p=0.02) in the serum of infertile women with family history group compared with infertile women without family history group (Table 3.16).

Table (3-16) The concentrations of parameters under study in sera of infertile women with and without a family history of infertility

Parameters	With family history No. = 10 Mean ± SE	Without family history No. = 23 Mean ± SE	P-value
HRG (ng/ml)	8.95 ± 3.26	31.79 ± 9.92	0.03
Inhibin B (pg/ml)	237.79 ± 35.18	243.76 ± 25.51	0.89
AMH (ng/ml)	6.29 ± 1.25	4.87 ± 0.82	0.35
Cu (µg /dl)	143.80 ±20.69	136.17 ± 11.07	0.72
Fe (µg /dl)	46.30 ± 12.21	86.78 ± 11.44	0.02
Zn (µg /dl)	86.70 ± 7.20	81.30 ± 6.27	0.57
Mg (Mg/dl)	3.46 ± 0.35	2.70 ±0.13	0.02
Cu/Zn	2.15 ± 0.4	3.16 ± 1.09	0.55

One of the many challenges of infertility is hearing the assumptions family; infertility can have an impact on one's relationships with family and friends [157].

The results of the present study agree with Mokhtar, *et.al*.(2006) study who found that 7.85% of infertile women have a positive family history of infertility among first-degree relatives [158].

3.6.4 Regulation of menstrual cycle

In this study, infertile women were classified into two groups: group1 is regular of menses (48.48%) and group2 is irregular of menses (51.51%) of infertile women (Fig. 3.9).

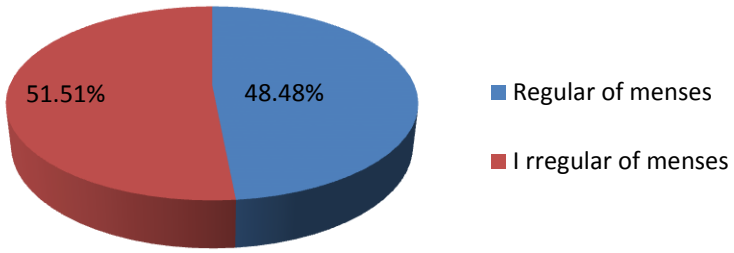


Fig. (3.9) The percentage of infertile women with regular and irregular of the menstrual cycle

The results did not show significant (P>0.05) differences in the concentration of all parameters under study between regular and irregular menses of infertile women (Table 3-17).

Table (3-17) The concentrations of parameters under the study of infertile women with regular and irregular menses

Parameters	Regular of menses No. = 16 Mean ± SE	Irregular of menses No. = 17 Mean ± SE	P-value
HRG (ng/ml)	26.53 ± 11.11	23.30 ± 9.52	0.82
Inhibin B (pg/ml)	232.90 ± 27.04	250.47 ± 31.10	0.67
AMH (ng/ml)	4.91± 0.84	5.66 ± 1.08	0.58
Cu (µg /dl)	136.87±14.87	140.00± 13.26	0.87
Fe (µg /dl)	76.50 ± 14.81	72.64 ± 11.87	0.84
Zn (µg /dl)	83.31± 8.39	82.58 ± 5.37	0.94
Mg (mg/dl)	3.06 ± 0.25	2.81 ± 0.17	0.40
Cu / Zn	2.34 ± 0.58	3.33 ± 1.40	0.52

The menstrual cycle is necessary for human reproduction. It prepares a woman's body for pregnancy through a number of synchronized events; it is regulated by combinational effects of hormones [159]. To our knowledge, there are no other studies investigating the joint effects of amount and duration of menstrual flow, or the effect of time to cycle regularity, on fecundability [160].

HRG, corresponding to the regular and irregular menses so as to plasma levels of HRG are lower in women who later develop preeclampsia in comparison with healthy pregnant women [161].

Once menses ensues, FSH levels begin to decline due to the negative feedback of estrogen and the negative effects of inhibin B produced by the developing follicle [162]. The major form of inhibin secreted during the follicular phase of the menstrual cycle is inhibin B. Serum levels rise sharply from the early follicular phase of the menstrual cycle, with a peak following the FSH rise and a progressive fall during the remainder of the follicular phase. Another peak of serum inhibin B is observed 2 days after the midcycle LH peak, followed by a rapid decrease and constant low levels during luteal involution [93].

Some have reported that inhibin B is useful in predicting ovarian response, whereas others have reported inhibin B does not add value to FSH, age or oestradiol. In the menstrual cycle, inhibin B levels progressively rise at peak concentration around days 5- 6 of the cycle, and found that the increase in inhibin B to ovarian stimulation is useful in predicting ovarian response [163].

Serum AMH levels are known to be a highly reliable marker for measuring ovarian reserve because they are not affected by gonadotropin, and AMH exhibits minimal variability within or among menstrual cycles [164].

Detailed counseling about the potential for a poor ovarian response and a high cycle cancellation is necessary for these young patients to reduce the psychological burden of treatment failure or cancellation [165].

Copper is associated with estrogen, women frequently develop premenstrual problems as estrogen levels will normally rise markedly prior to menstruation. An increase in estrogen levels will exacerbate an existing hormonal imbalance in these women with symptoms similar to those of copper toxicity; frontal headaches, constipation, fatigue, depression, volatility, weight gain, and food cravings [166]. The role of copper in human reproduction has not been much investigated [120].

Many authors have assumed that menstrual loss of iron is an important aetiological factor in iron deficiency. Iron deficiency is not uncommon in women with a history of heavy menses. Ovulation is followed by the luteal phase of the cycle when progesterone levels increase in order to prepare the uterus for a possible pregnancy [167].

Zinc also plays an important role in sexual development, ovulation, and the menstrual cycle in females and shows reducing complications in pregnancy [101].

The effect of menstrual cycle phases on the indexes of magnesium and zinc status was assessed in five normally menstruating women. Plasma concentrations of magnesium and zinc and the magnesium and zinc content of red blood cells and peripheral blood mononuclear cells were measured for 3 or 5 days per week during three menstrual cycles. The cycles were divided into four phases: menses, follicular, ovulatory and luteal. Plasma magnesium concentrations were highest during menses and then gradually declined, reaching the lowest point during the ovulatory phase. This was followed by a rise during the luteal phase. Plasma zinc concentrations were higher during menses and the follicular phase and then dropped during the ovulatory and luteal phases [168].

Magnesium is also critical for those struggling with premenstrual, including premenstrual migraines, irritability, low mood, and cramps [169].

For now, it seems more than reasonable to increase intake of magnesium for all individuals especially those being managed for menstrual irregularities and Premenstrual Syndrome. Pandya, *et al*. (1995) revealed that the levels of serum magnesium were highest during the luteal phase and lowest during the follicular phase. This elevated metabolism requires magnesium ions and oxidative enzymes which were found to be increased significantly during the luteal phase [170]. Mg, as a result, may influence endometriosis through its effect on retrograde menstruation [171].

3.6.5 Duration of infertility

In this study, by using Pearson's correlation coefficient, the results showed that there was a positive significant (r= 0.59, p < 000) correlation between Fe and duration of infertility whereas no significant (p > 0.05) correlation in a concentration of other parameters under study and duration of infertility (Table3.18). Also, the results showed that there is a linear relationship between duration and iron (Fig. 3.10).

Table (3-18) Correlation between parameters under study and duration of infertility

Parameters	r	P-value
HRG (ng/ml)	- 0.03	0.87
Inhibin B (pg/ml)	- 0.31	0.07
AMH (ng/ml	- 0.01	0.94
Cu (µg /dl)	- 0.05	0.75
Fe (µg /dl)	0.59*	0.000
Zn (µg /dl)	0.09	0.61
Mg (Mg/dl)	- 0.19	0.28
Cu ⁄ Zn	- 0.10	0.56

***Correlation is significant at the 0.01 level (2-tailed)**

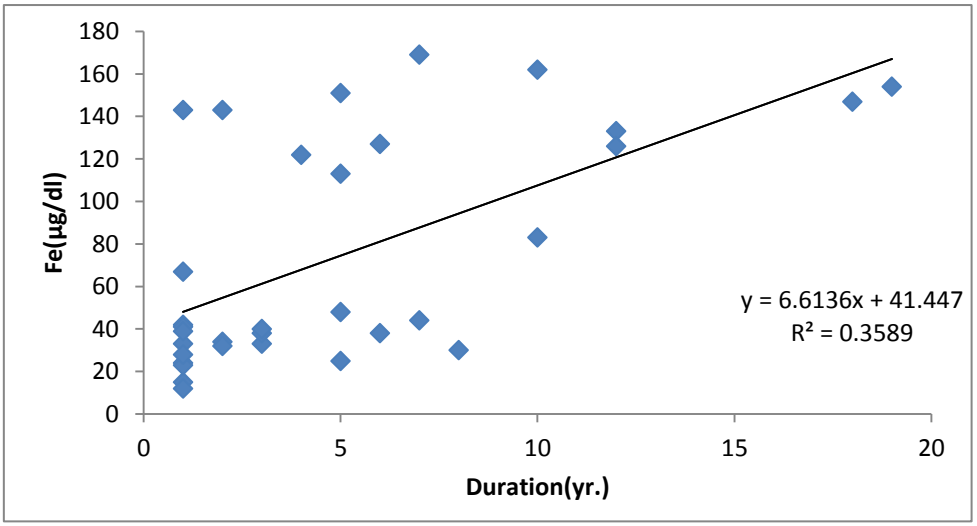


Fig. (3.10) The correlation between duration and Iron of infertile women

The duration of infertility should be considered as a factor that may affect the outcome of ART. It is unclear the effect of duration of infertility on the outcome of infertility treatment. As the duration of infertility increases, the couples become less interested to seek the medical services. Besides, the psychological factors depression and hopelessness play a major role in these cases [172].

Galliano and Pellicer, (2015), stated that duration of infertility being the most important prognostic factors that similar observations were found in different infertile populations, based on the duration of infertility [173].

The important studies have attempted to establish the relative importance of duration of infertility in predicting the likelihood of live birth in infertile couples, without treatment [174].

3.6.6 Abortion

In this study, infertile women were classified into two groups: group1 is infertile women who have an abortion (30.30%) and group2 is infertile women that don't have an abortion (69.69%) (Fig.3.11**).**

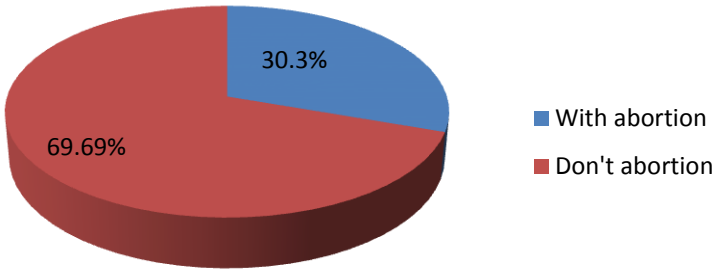


Fig. (3.11) The percentage of infertile women with and don't abortion

By using Pearson's correlation coefficient, the results did not show a significant correlation between the parameters under study and abortion of infertile women (Table 3. 19).

Table (3-19) Correlation between parameters under study and abortion of infertility

Parameters	r	P-value
HRG (ng/ml)	0.23	0.31
Inhibin B (pg/ml)	- 0.14	0.54
AMH (ng/ml)	- 0.003	0.99
Cu (µg /dl)	- 0.27	0.19
Fe (µg /dl)	- 0.05	0.80
Zn (µg /dl)	0.27	0.20
Mg (µg /dl)	- 0.17	0.41
Cu / Zn	- 0.19	0.28

Abortion is a terminating an unintended pregnancy either by individuals without the necessary skills or in an environment that does not conform to minimum medical standards, or both [175].

A missed miscarriage is a type of abortion in which the fetus dies but the fetal tissue is not expelled by the woman's body and remains there until it is removed by a doctor [176]. The experience of repeated pregnancy loss is physically and emotionally traumatic for women who are trying to have children [177]. Recurrent miscarriage is defined as two or more miscarriages, affects approximately 5% of all couples, Jenkins, *et al*. (1991) found that women with poor ovarian reserve that do proceed to follicle aspiration produce low numbers of oocytes and embryos and have low pregnancy rates and high miscarriage rate: Women with unexplained recurrent miscarriage feel more compelled to return the survey than women with a known cause of their recurrent miscarriage [178].

The magnitude of the effect of induced or spontaneous abortion or of their combination may be assessed in terms of the risk ratio (relative risk). This is the ratio of the risk of secondary infertility among women with one or more induced or spontaneous abortions or any combination of them to that among those without any induced or spontaneous abortion [52]. Therapy has been shown conclusively to reduce miscarriage rates, although some studies have indicated that these are potentially promising treatments [179].

Studies indicate that women with low estrogen and low copper have more miscarriages. This is important for some women to know. Controlling the copper imbalance can help immensely with normal pregnancy. On the other hand, infertility is more common among women with elevated or biounavailable copper. This may be due, in part, to weak adrenals that, in turn, give rise to copper imbalance [107].

The social stigma attached to abortion may have limited the number and/or types of abortions reported. Despite this, there was a positive correlation between the number of spontaneous abortions and the likelihood of developing anemia at final Hb reading [180].

Zinc is a crucial component of DNA and it's deficiency can cause chromosome changes which leads to infertility and an increased risk of miscarriage [106]. Zn deficiencies have been associated with abortion, fetal mummification, lower birth weight and an improvement in conception rate at first service prolonged labor. It plays an important role in uterine [180].

Magnesium is needed for more than 300 biochemical reactions in the body. It helps to maintain normal muscle and nerve function, supports a healthy immune system, regulates blood sugar levels, promotes normal blood pressure, and is known to be involved in energy metabolism and protein synthesis [175].

Conclusions and Future studies

Conclusions

1. Infertile women are associated with the decrease level of inhibin B and Zn, whereas there is an increase in the magnesium level.
2. Inhibin B is considered a good parameter for predict infertility than AMH.
3. HRG, AMH, Zn, and Mg are associated with letrozole and gonadotropin treatment, as there is an increase in the level of HRG and decrease in the level of AMH, Zn and Mg in infertile women group after treatment.
4. Young women have higher risk of infertility than old women.
5. BMI, family history, regular menses, duration of infertility and abortion are considered secondary factors in the effect on levels of glycoproteins and trace element in infertile women group.
6. Most of the infertile women have irregular menses.
7. Increasing the size and number of oocyte after treatment in infertile women.

Future studies

1. Genetic study of HRG in infertile women.
2. The comparative study is needed to compare between two types of infertility before and after treatment.
3. Studying the correlation of infertility with the other diseases for example diabetes and depression.
4. Studying other trace elements which have a correlation with infertility like selenium and manganese.

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الخـــلاصـــــــة

مقدمة موجزة : العقم هو مرض يصيب عادة الجهاز التناسلي عند النساء ،فالعقم هو فشل حصول الحمل بعد مرور 12 شهراأو أكثر من الجماع الجنسي المنتظم .

لقد ازداد العقم في السنوات القليلة الماضية ذلك ان العقم في العراق لا يعد مشكلةَ صحيةَ ناتجةَ عن تشوه الرحم أو التهابات في قناة فالوب فقط إنما هناك العديد من الأسباب التي يمكن ان تؤدي إلى حصوله كالاسباب البيئية و الاجتماعية والاقتصادية.

الغرض من الدراسة : تهدف هذه الدراسة إلى التحري عن تأثير بعض المتغيرات الكيموحيوية وعلاقتها بالعقم .

العينات وطرائق العمل : تضمنت الدراسة 33 امرأةَ عقيمةَ تراوحت أعمارهن بين (15-44) سنه و 22 امرأة غير عقيمة (كمجموعة سيطرة) . وقد أخذت العينات من العيادة النسائية الخارجية للتوليد وتم قياس كل من HRG inhibin B , AMH في السيرم للنساء العقيمات ، وغير العقيمات بوساطة تقنية (ELISA) ، فضلا عن انه تم تحديد العناصر الضئيلة مثل Cu, Fe, Zn, Mg بوساطة طرائق الطيف.

وقد أجري الجزء العملي لهذه الدراسة في المستشفى الحسيني التعليمي في كربلاء ابتداءً من شهر تشرين الأول 2016 وانتهأءَ شهر نيسان 2017.

النتائج :ا ظهرت النتائج الإحصائية عن وجود انخفاض معنوي واضح ($p = 0.05$) في تركيزinhibin B في مصول النساء العقيمات مقارنة مع مجموعة السيطرة(النساء غير العقيمات) فضلا عن انخفاض معنوي في تركيز الخارصين وارتفاع معنوي ($p<0.000$) ملحوظ في تركيز المغنيسيوم للنساء العقيمات مقارنة مع مجموعة السيطرة ، إلى ما بين ذلك أوضحت النتائج وجود ارتباط معنوي بين النحاس والمغنيسيوم $r=0.48$, $p=$ (0.000) بين المجموعتين.

من جهة أخرى فقد أظهرت نتائج النساء العقيمات من النوع الأولي وجود ارتباط معنوي موجب واضح بين المغنيسيوم و inhibin B ($p=0.03,r=0.58$) وارتباط معنوي سالب بين النحاس والحديد ($p= 0.03, r= -0.54$).

أما ما يتعلق بنتأئج النساء العقيمات من النوع الثانوي فقد أظهرت النتائج وجود علاقة سالبة بين HRG والمغيسيوم ($r=-0.43$, $p=0.05$) بين المجموعتين.

وتشير النتائج إلى وجود انخفاض معنوي واضح (p=0.01, p=0.04) في تركيز HRG,AMH في النساء العقيمات بعد العلاج مقارنة مع النتائج التي اجريت لهن قبل العلاج، وايضاَ فقد بينت النتائج انخفاضآ معنوياً واضحآ (p=0.000,p=0.004) بين تركيز الخارصين و المغنيسيوم في النساء العقيمات بعد العلاج مقارنة مع النتائج قبل العلاج.

وقد كشفت النتائج وجود ارتباط معنوي موجب (p=0.000 ,r=0.71) بين الحديد وعمر النساء العقيمات .

وكشفت نتائج الدراسة أيضاَ عن وجود انخفاضَ معنوي ملحوظ (p=0.03) في تركيز HRG للنساء العقيمات اللواتي لهن تاريخاَ عائلياً في العقم مقارنة مع اللواتي ليس لديهنٌ حالات عقم في تاريخ عوائلهنَ . وظهر من خلال الدراسة وجود انخفاض واضح (p=0.02) في تركيز الحديد عند النساء العقيمات اللواتي لديهن حالات عقم قي تاريخ عوائلهنٌ مقارنة مع اللواتي ليس لديهن حالات عقم في عوائلهن وايضاَ بالنسبة لتركيزالمغيسيوم (p=0.02) .

أخيراَ فإن الدراسة توصلت إلى وجود ارتباط معنوي موجب (p <0.000, r=0.59) بين الحديد ووقت حصول العقم . بينما لا يوجد أي ارتباط معنوي بين العوامل الأخرى قيد الدراسة.

الاستنتاج : تشير النتائج إلى ارتباط عقم النساء بنقصان مستوى inhibin B و الخارصين مع زيادة مستوى المغنيسيوم ، فضلاً عن إشارة النتائج الى inhibin B وهو مؤشر سريري جيد يمكن أن يسٌتدل به عن العقم عند النساء ويمكن اعتماد inhibin B كمؤشر جيد للتنبؤ بالعقم بدلاً من AMH.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية العلوم

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رسالة مقدمة الى
مجلس كلية العلوم جامعة كربلاء
وهي جزء من متطلبات نيل درجة الماجستير في
الكيمياء الحياتية

من قبل

انعام جوده راضي

بكالوريوس علوم كيمياء / جامعة بابل (1996)

بإشراف

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دكتورة النسائية والتوليد
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بورد في النسائيه والتوليد والعقم

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