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A study of fertility hormones in women with recurrent miscarriages in Kerbala city

A Thesis

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Dedication

To my father who taught me everything and expected nothing in return, to my mother who taught me the meaning of sacrifice and devotion, for my beautiful sisters she helps me when i need it, to my husband for his support, encouragement and love, to my pride and joy my children (Joud & Naz), and to everyone who supported me, even with a smile.

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Summary

This study was designed to investigate the relation between recurrent miscarriages and fertility hormones with the presence of some major causes of miscarriage such as polycystic ovary syndrome, obesity and maternal age. The study included 90 women, aged between (15-45)years, divided into 50 patients women with polycystic ovary syndrome and suffering from recurrent miscarriage and 40 women as a healthy control group. The samples were taken from the Gynecological and Obstetric Teaching Hospital in Kerbala and from the gynecological outpatient clinics during the period from 1/10/2021 (October) to 9/29/2022 (September), where the fertility hormones were measured, FSH ,LH, ESTROGEN, prolactin, progesterone and testosterone were measured by ELISA technology and using the CL-900i device from mindray Company in China, and measurement of the hormones INHIBIN B and ACTIVIN A by ELISA technology is from BioTek in USA. The results of the statistical analysis found that there was a significant difference in ACTIVIN A,INHIBIN B,FSH,LH, ESTROGEN, prolactin, progesterone and testosterone in patients women compared to the control group, A decrease in the level of the hormones INHIBIN B and ACTIVIN A in serum of patients women with polycystic ovary syndrome in all age groups from (15-45)years compared to the control group, and an increase in the level of FSH, LH, ESTROGEN, prolactin, progesterone and testosterone in patients women for age (15-29)years compared to the control group, while we noticed a decrease in the level of some hormones in older women from (30-45) years, such as FSH, progesterone and testosterone compared to the healthy control group, and show that women who suffer from recurrent miscarriage due to polycystic ovary syndrome had a higher body mass index (BMI) with a percentage of 39%, compared to overweight women with a percentage of 34%, and women of normal weight with a percentage of 27%. The results of the study also

showed a difference in the levels of fertility hormones concentration with the duration of miscarriage, the number of recurrent miscarriages and the date of the last miscarriage in patients women with PCOS.

List of Content

List of Figure

List of Table

List of Abbreviation

The Term	Definition
ºC	Degrees Celsius
m²	Square meter
µl	Microliter
r	Correlation coefficient
ASRM	American Society of Reproductive Medicine
ABEI	Monoclonal Antibody
BMI	Body Mass Index
BMP	Bone Morphogenetic Protein
CAH	Congenital Adrenal Hyperplasia
DHEA	Dehydroepiandrosterone
E2	Estradiol
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immuno Sorbent Assay
FS	Folliculo Stellate
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
HPG	Hypothalamus-Pituitary-Gonadal axis
IVF	In Vitro Fertilization
INHBB	INHIBIN B
K	Kilo
Kg	Kilogram
LH	Luteinizing Hormone
mm	Millimeters
ml	Milliliter
ng	Nanograms
nm	Nanometer
NIH	National Institutes of Health Optical Density
OD	Optical Density
Pg	Picogram
PRL	Prolactin
POF	Premature Ovarian Failure
POI	Premature Ovarian Insufficiency
PCOS	Polycystic Ovary Syndrome

P-value	Probability level of statistical
RLUs	Relative Light Units
SD	Standard Deviation
SA-HRP	Streptavidin-Horseradish Peroxidase
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone
TMB	Tetramethylbenzidine
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

AND

LITERATURE REVIEW

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Recurrent Miscarriages

The expression miscarriage refers to the loss of a pregnancy before the embryo reaches viability. As a result, the term encompasses all miscarriages from conception until 24 weeks of pregnancy. Recurrent miscarriage, It is described as the loss of two or more consecutive pregnancies, impacts 1% of couples trying to become pregnant. 1-2% of patients of first- and second-trimester pregnancies miscarry before the 24-week stage [(1)]. Infertility and miscarriage are two types of reproductive failure they have almost the same reasons, according to experts. Polycystic ovary syndrome disease, uterine septum, and uterine fibroid are just a few of the diseases, which are related to both infertility and miscarriage. Patients who have had a recurrent miscarriage have a higher likelihood of infertility [(2)].

1.1.1 Causes of Recurrent Miscarriages

There are many causes of recurrent miscarriages:

1. It is a genetic defect in one or both couples that accounts for (3-5%) of the reasons of recurrent miscarriage [(3)].

2. Hormonal reasons: A less of progesterone secretion is one of the most common causes of recurrent miscarriage, accounting for 10-15% of the causes. Only in these conditions , such as cases of diabetes out of control by drugs, well-known stabilizing methods such pills, suppositories, progesterone injections, or hormones produced by the pituitary gland are all possible. Diabetes, whether managed by insulin injections raises the risk of miscarriage and congenital malformations in the embryo, as well as ovarian syndrome, which is accompanied by an increase in the LH hormone, which relates to infertility and recurrent miscarriages, and

ovulation disorders, which result in eggs that are immature, malformed, or incompatible with the uterine lining [4].

3. An abnormality in the uterus's anatomy: Genetic defects like the uterine septum can be the source of 10-15% of the causes of recurrent miscarriage. It may be discovered with a vaginal ultrasound and is responsible for 70% of the causes of anatomical malformations. The uterine septum, which changes the uterus's shape, is the root of this problem. Furthermore, there aren't enough capillaries there to maintain the pregnancy . The disease is usually treated by surgery [5].

4. Immunity related causes : The mother's body must respond in order to accept this foreign part without attacking or rejecting the unborn child because it occurs in 3–4% of cases. The immune system in the woman's body plays a role in stopping this interaction with so inhibition of antibodies, and the growing embryo is taken into account whether this system has any deficiencies. Regular miscarriages are a problem that many women face [6].

5. Bacterial or viral infections: Miscarriage can occur as a result of any serious infection, whether bacterial or viral, such as Toxoplasmosis or German measles [7].

1.1.2 Symptoms of Recurrent Miscarriages

The symptoms and indicators of spontaneous miscarriage differ depending on the type. Miscarriage may be asymptomatic or result in the return of natural pregnancy symptoms and signs. Miscarriage whether threatening or no are linked to abdominal-pelvic cramps, vaginal bleeding, fever, secretions of cervical or vaginal, tachycardia, and hypotension. It is a good idea to try to gauge the volume of bleeding because more than typical menses can suggest a miscarriage. Patients with significant bleeding may develop symptoms and signs of a

miscarriage, the gestational age and position of the pregnancy should be assessed using the beginning day of the last menstrual cycle and findings from any previous ultrasounds. Finally, a pelvic exam is crucial in determining the cause of a possible miscarriage [8].

1.1.3 Risk Factor of Recurrent Miscarriages

It is difficult to determine the cause of recurrent miscarriage; it is a very stressful situation for both partners and doctors. Pregnancy loss is a common occurrence, pregnancy losses in the first trimester are more common than in the second trimester. Early pregnancy loss can be caused by a variety of conditions, including advanced mother age, advanced father age, smoking, and alcohol intake. Immunological and genetic alterations were among the other factors. In both the affected couple and the embryo, genetic alterations are commonly described as chromosomal abnormalities. In order to rule out chromosomal translocations, a genetic test is performed for the husband, as well as maternal testing for thyroid problems (endocrine) and anti-phospholipid antibodies (autoimmune), endometrial or uterine cancer [9]. Recurrent miscarriage is a complex disorder that involves relationships between female and male, and cumulative (placental / fetus) risk factors in pregnancy development and continuation systems [10].

The most common reason of recurrent miscarriages is embryonic chromosomal abnormalities, which are linked to women's age when they delay childbearing until their late 30 year or early 40 year. Although maternal age is a risk factor for recurrent miscarriage, other variables are more important, because the likelihood of an early pregnancy loss due to severe chromosomal alterations decreases as the number of miscarriages. There is a two to triple rise in the rate of spontaneous abortion in women over 40 year who attempt pregnancy , it

also increase the risk of genetic abnormalities [11]. Obesity is now recognized as a risk factor for miscarriage on its own. Obesity before pregnancy has a significant impact on the rate of pregnancy and abortion [12]. A uterus with an abnormal anatomical structure is a risk factor for spontaneous abortion. The uterine septum and uterine fibro may alter the uterus cavity's natural anatomy and local environment, leading to complications. Increased blood flow resistance, decreased embryo implantation area and endometrial blood flow as a result, embryo implantation, growth, and embryo development are affected the disease is a kind of endometriosis is a risk of complications of pregnancy and childbirth, as it causes inflammations in the body, damage to the structure of the uterus, in addition to some hormonal effects, and all of this causes miscarriage. Some research has also suggested patients with polycystic ovary syndrome (PCOS) have a greater chance of spontaneous abortion [13]. As a result, abortion is not just a health issue that costs society a lot of money because it causes so many difficulties, but it is also a social issue in the community. Because it, in the end puts people's health in a danger [14]. The findings of numerous investigations There is a variety of abortion-related issues that can arise women's personal lives, and they came to the conclusion that six weeks following an abortion, do women's feelings change, and pregnancy termination, both of which have an impact on women psychologically Feelings of emptiness and remorse, Moreover, women who have a spontaneous Abortion carries a double risk of severe depression. In most cases, this begins within the first week after an abortion. It is more severe in women [15]. Because recurrent miscarriage is typically thought of as a feminine disease, women who suffer from it face greater familial and societal issues than males. Recurrent miscarriage can lead to psychological suffering, family threats, remarriage, separation, and divorce [16].

1.2 The Main Causes of Recurrent Miscarriage

1.2.1 Polycystic Ovary Syndrome

PCOS (polycystic ovary syndrome) is a prevalent endocrine condition that affects women of reproductive age [17, 18]. The prevalence of PCOS in women of reproductive age ranges from 5% to 18%, according to a systematic review and meta-analysis [19]. High levels of androgens (hyperandrogenism), ovarian dysfunction, and polycystic ovaries are all symptoms of PCOS [20]. This multi-factorial condition first manifests throughout puberty [21]. Women with PCOS are at risk for fertility issues (menstrual cycle disorders, failure to ovulate, late menopause, endometrial cancer, and infertility), metabolic issues (insulin resistance, diabetes type 2, hypertension, and cardiovascular diseases), physical issues (central obesity, acne, hair loss, and baldness), and psychological issues (depression, stress and anxiety) [22]. Menstrual dysfunction and clinical or laboratory high androgen level are the two main components for diagnosing this condition, and these elements are employed in clinical diagnosis [23]. Most PCOS individuals only have one or two clinical symptoms. Menstrual disorders are the most prevalent clinical finding, which generally begin at or shortly after menarche and can show as hypo menorrhea, amenorrhea, or poly menorrhea, until the menstrual cycle is regular [24].

The research suggests that this syndrome is a state that showed in adolescents because of inherited ovarian dysfunction to androgen secretion that is excessive, and there is evidence that PCOS has a hereditary foundation. When the embryo existence and physiologically ovary stimulation by the hypothalamus-pituitary axis during the birth period and at the start of adolescence [25]. Changes in the concentrations of luteinizing hormone (LH), ESTROGEN, prolactin, and serum

androgens such as testosterone are often linked with PCOS. Many women with PCOS have an elevated LH/FSH ratio, according to hormonal measurements [26]. A number of organizations has published diagnostic criteria for this condition. The National Institute of Health The National Institutes of Health (NIH) developed the NIH criteria. The American Society of Reproductive Medicine (ASRM) has issued a statement. The Rotterdam criteria have been developed, as well as the Androgen criteria Society for Excess and Polycystic Ovary Syndrome [27, 28].

The polycystic ovary is bigger, has more follicles, and has a particularly thick core tissue where testosterone produced, compared to the normal ovary. The normal ovary has five follicles on average and is around the size of a walnut. The polycystic ovary has 10 or more follicles, which are usually tiny follicles measuring two to ten mm in diameter. Polycystic ovarian cysts are normally the size of a hen's egg, but they can also reach the size of an orange. The increased size of the polycystic ovary is primarily due to an increase in tissue, rather than, as one might assume, because of increased ovarian size of the cysts or additional follicles, the follicles are usually too tiny to make a significant contribution to the size of the ovary [29].

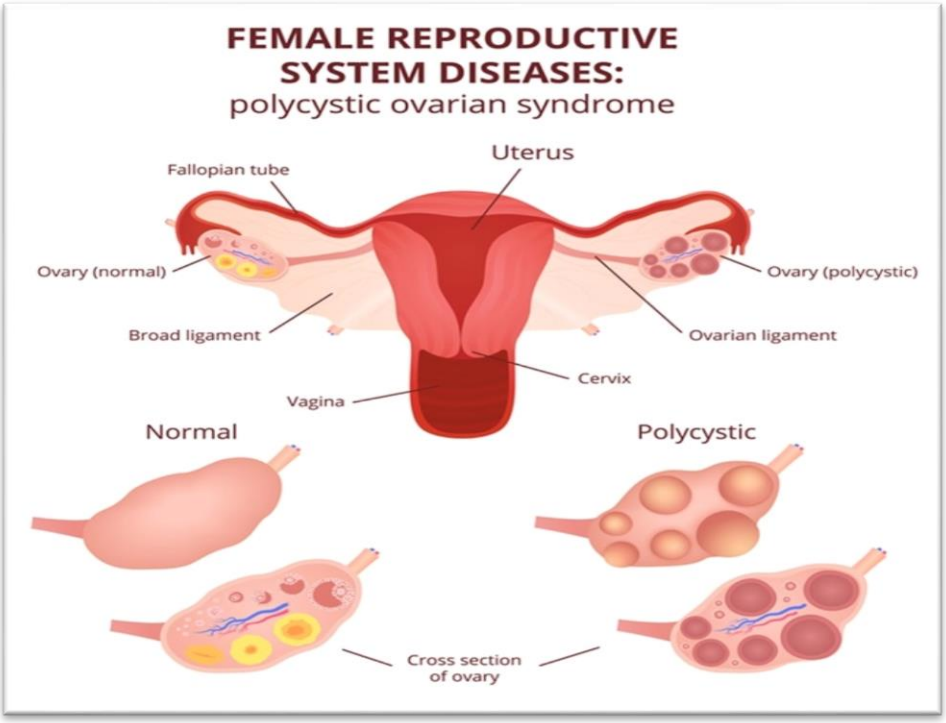

Figure 1.1 Female reproductive system, the uterus and ovaries scheme, PCOS [30].

1.2.1.1 Types of PCOS

PCOS can be divided into four types based on a variety of factors:-

1. PCOS (insulin-resistant polycystic ovary syndrome).
2. Adrenal PCOS is a kind of PCOS that affects the adrenal glands.
3. Thyroid dysfunction in polycystic ovarian syndrome patients.
4. PCOS that is inflammatory.

1. Insulin resistance is defined as a high amount of insulin hormone: Metabolic syndrome is sometimes known as pre-diabetes. Measuring the hormone insulin is the best approach to check for insulin resistance. Insulin resistance and compensatory high level of insulin affects between 65 and 70 percent of female with polycystic ovary syndrome [31].

2. Adrenal PCOS is a kind of PCOS that affects the adrenal glands: Adrenal androgen excess in PCOS may result from adrenal hyperplasia, adrenal androgen-producing cancer, Cushing's syndrome, or genetic

adrenal androgen production [32]. About a quarter of all circulating testosterone is produced in the adrenal cortex. Excess adrenal androgen in PCOS could indicate hypothalamic-pituitary dysfunction, adrenocortical abnormalities, or cortisol metabolic abnormalities in PCOS hepatic tissue [33].

3. Thyroid dysfunction in polycystic ovarian syndrome patients: As shown in figure, In primary hypothyroidism, elevated (TRH) hormone causes elevated prolactin and thyroid stimulating hormone (TSH). Increased TSH inhibits ovulation as a result of a change in the ratio of follicle stimulating hormone (FSH) and luteinizing hormone, which has effect on FSH receptors and leads to polycystic ovarian shape. Hypothyroidism has also been linked to increased collagen deposition in the ovaries [34].

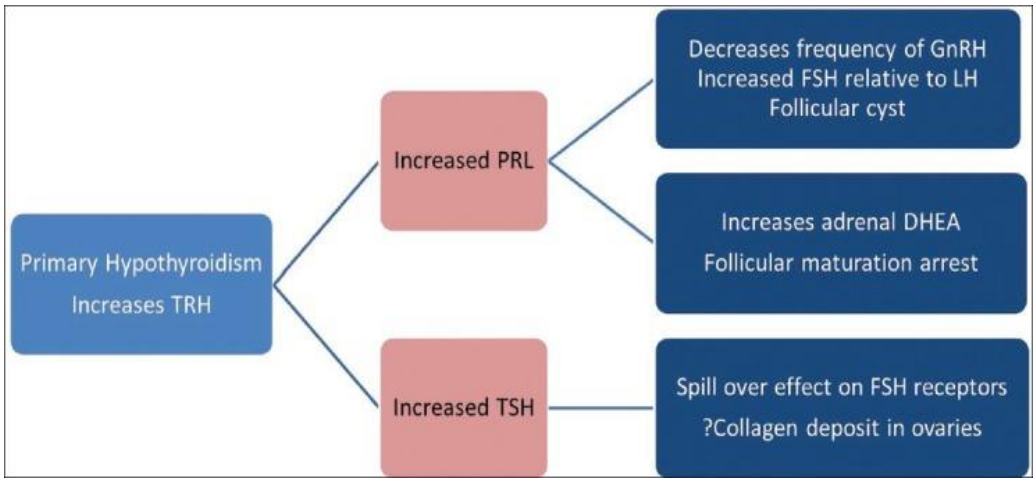

Figure 1.2 Primary hypothyroidism in patients with polycystic ovary syndrome: Pathophysiology [35].

4. PCOS that is inflammatory: Chronic inflammation caused by chlamydial infections can result in disease processes that lead to metabolic and hormonal problems, resulting in PCOS [36].

1.2.2 Obesity effect

One of the biggest risk factors for reproductive outcomes is pre-conception weight, and it is widely known that weight loss enhances fertility in overweight and obese women [37, 38]. Clinically obese women have a body mass index of greater than 30 kg/m^2 [39]. Women who are overweight or obese experience natural menstrual cycle disturbances at about three times the rate of women who are healthy weight. In women who are overweight or obese, there is also a link between their pre-conception Mass index and the time it takes to fall pregnant [40]. Obese women may find it difficult to conceive since they are overweight prior to conception. As a result, many women seek help from healthcare specialists. Many overweight women who are having difficulty conceiving have co-morbidities such as polycystic ovarian syndrome (PCOS), which can complicate fertility due to insulin resistance, sex-steroid metabolism, and menstrual cycles. PCOS is thought to affect 75 percent of infertile overweight or obese women [41]. Although assisted reproductive technology, such as in vitro fertilization, can help these women and other infertile couples become pregnant, it is not a foolproof method [42].

1.2.3 Maternal age

It is commonly understood that female fertility reduces with age, resulting in an increase in miscarriage. Recurrent miscarriages share risk factors with a variety of other reproductive problems. According to studies, maternal age is positively related to the number of miscarriages in women who have them often [43]. It has a role in predicting the risk of a miscarriage [44]. The pregnancy rate of women with recurrent miscarriages in vitro fertilization (IVF) therapy decreased as their maternal age increased [45]. The number and quality of remnant oocytes

decrease as maternal age increases [46]. Previous pregnancy history is an important predictor of future pregnancy outcome. After each pregnancy loss the probability of another miscarriage rises reaching around 40% after three consecutive pregnancy losses and the prognosis worsens as the mother's age rises [47]. At increasing maternal age, the probability of miscarriage due to chromosomal abnormalities of the embryo increases. It's important to note, that as the miscarriages rises, so does the risk of pregnancy loss [48].

1.3 Fertility hormones

The menstrual cycle is a natural phenomenon that marks the years of fertility in women's life. It is a sign of reproduction and menstruation, as well as a signal of Women's health is important. The majority of women at this point are in pain. Due to hormonal fluctuations, you may experience a variety of symptoms [49]. Hormonal changes related with menstruation problems, such as low and high hormone concentrations, have significant consequences on the body [50]. The ovaries secrete estrogen, progesterone, and the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) after a girl reaches reproductive age. The buildup and breakdown of these hormones are determined by their rise and fall. During the menstrual cycle, the interior layer of the uterus sheds, as well as the growth and development of the uterus the laying of an egg Pregnancy is made possible by this mechanism [51]. From a biological viewpoint, the best age for women to have children is between the ages of 18 and 30 [52]. Female fertility begins to drop after the age of 30, with lower pregnancy rates per cycle and eventual infertility [53].

1.3. 1 Luteinizing hormone (LH)

Luteinizing hormone (LH) is an important gonadotropin in the reproductive system's control. LH enhances progesterone secretion in the luteal phase and started oocyte maturation by stimulating the generation of sex steroids [54]. LH is produced in the anterior pituitary gland, and its receptor is found in the gonads, where it performs these reproductive tasks [55]. LH is a pituitary hormone that stimulates the corpus lutein and causes ovulation. During the second half of the cycle, the ovary secretes progesterone and estrogen cycle of menstruation [56]. The release of the hormones LH and FSH, which usually starts towards the start of the cycle, promotes a rise in estradiol (E2) production from the ovaries between days 8 and 12 of the cycle (before ovulation). Around day 14, there is an increase in progesterone, and around day 18, there is a surge in estrogen (after ovulation). When a woman uses hormonal contraception. Progestin inhibits FSH release early in the cycle, preventing the onset of menopause. E2 increases naturally, causing a surge in LH and so preventing ovulation [57].

In women, LH helps FSH stimulate follicles and it also has a significant impact on ovulation and sperm production the ovum's release, This is accomplished by a significant increase in LH caused by the positive feedback activity of increasing estrogen on the pituitary gland, which is responsible for the completion of meiosis in the main oocyte, follicular rupture wall, followed by ovulation 9 hours later LH levels at their highest point. LH promotes progesterone secretion in the postovulatory phase, which then forms maintains the ovarian follicles. The ovarian follicles is a tiny yellowish hormone secreting structure that is created from the remains of the ovary. The follicle once housed the growing follicle ovum. It works by releasing tremendous amounts of

energy estrogen, progesterone, and tiny quantities of progesterone which are necessary for the installation and preparation for the purpose of pregnancy [58].

Low levels of human LH and FSH may be an indication of pituitary failure, but gonadal failure may be indicated by elevated levels of LH and FSH, as well as lower levels of gonadal steroids (menopause , ovary removal , premature ovarian syndrome) [59]. LH is a hormone with a molecular weight of 28,500 Dalton [60]. It is a heterodimer made up of two subunits and one LH-specific component [61]. There are 92 amino acids in the subunit α, with 5 disulfide bridges as well as an N-linked carbohydrate site [62]. LH has 121 amino acids, six disulfide bridges, and two N-linked carbohydrate sites in its subunit β [63].

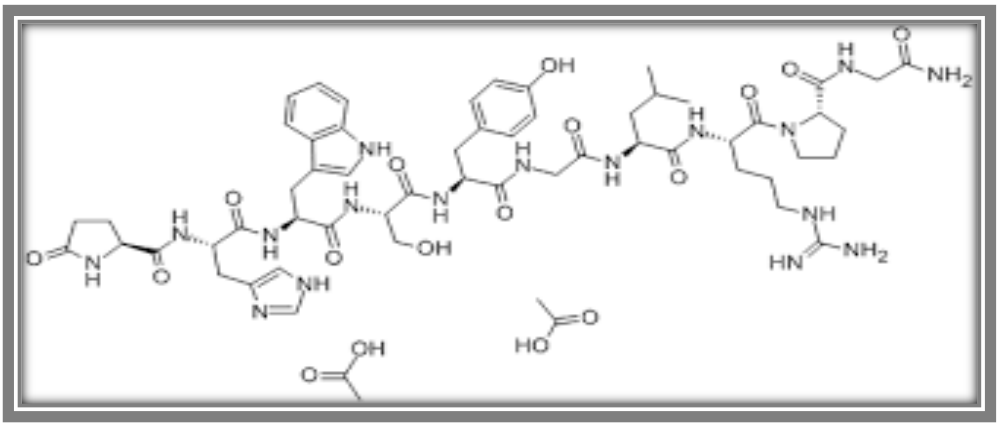

Figure 1.3 Luteinizing hormone Structure [64].

1.3. 2 Follicle stimulating hormone (FSH)

FSH is a type of gonadotropin that is released by the anterior pituitary's basophilic cells and plays a key role in gonadal hormone synthesis and reproductive process regulation [65]. Gonadotropin releasing hormone is produced largely from hypothalamic neurons and reaches the anterior pituitary, where it is coupled with Gonadotropin releasing hormone receptor to regulate FSH secretion in the

hypothalamus-pituitary-gonadal (HPG) axis [66].The ovarian follicle-stimulating hormone (FSH) increases the formation and development of ovarian follicles, where ova or egg cells develop and the ovaries secrete estrogen. The best predictor of FSH-based ovarian reserve is maximum FSH [67].

The most frequent reason of increased serum FSH levels in women is menopause. gonad normal limiting feedback results in elevated levels of follicle stimulating hormone, which causes uncontrolled pituitary FSH production. If there are continuing elevated FSH levels during the reproductive years, this is abnormal. High FSH levels have been associated with several conditions, including early menopause (also known as premature ovarian failure), insufficient ovarian reserve (also known as premature ovarian aging), gonadal digenesis, and some types of congenital adrenal hyperplasia (CAH) [68].

Follicle stimulating hormone promotes follicular development, follicular cell proliferation, androgen aromatization to estrogens, as well as LH receptor expression. LH is also required for follicle development, especially in the late stages [69]. FSH travels through the bloodstream to the gonads, where it stimulates follicle formation in females and spermatogenesis in males. FSH has an evident effect on the physiology of both sexes' reproductive systems [70]. FSH is made up of two subunits: alpha (which other glycoprotein hormones share) and subunit beta (it only applies to FSH) [71]. Follicle-stimulating hormone's molecular structure FSH has a molecular weight of 35 k Dalton and is made up of 92 and 111 amino acid subunits [72].

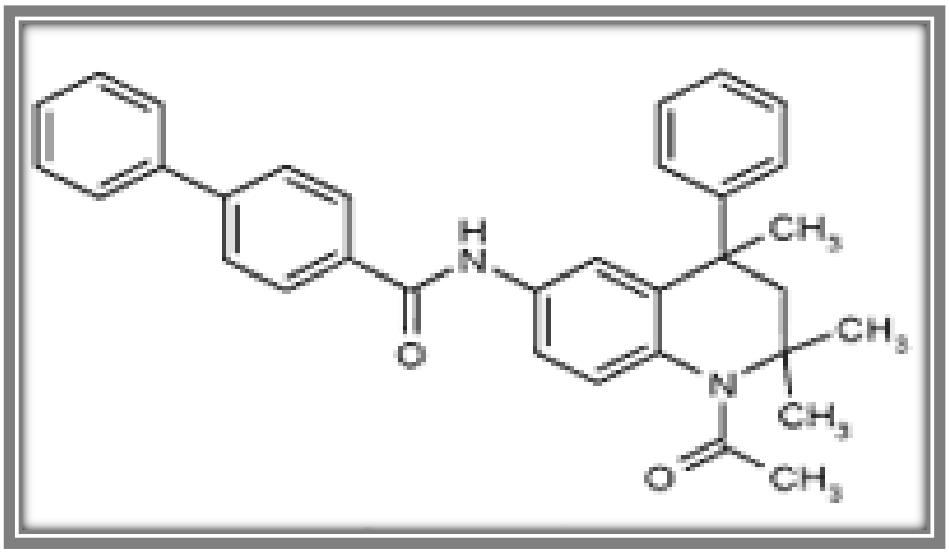

Figure 1.4 Follicle stimulating hormone structure [73].

In females, FSH is required for estradiol production, as well as growth and maturation Humans, for example, are mono-ovulatory. FSH promotes the stimulation, development, selection, and maturity of follicles, resulting in the production of only one kind of egg. During the moment of ovulation, one mature oocyte [74].The average human menstrual cycle lasts between 21 and 28 days. It is broken down into three sections. The follicular or proliferative phase is the first of the three phases [75]. Second ovulation, and then third the luteal or secretory phase [76]. The follicular phase lasts until ovulation and begins on the first day of menses. This phase lasts 7-17 days and is marked by a drop in body temperature and more crucially, the completion of the dominant follicle's development. Due to the expansion of the dominant follicle, hormonal changes such as increased release of estradiol and INHBB occur during this period [77].

FSH levels rise and fall as a result of this. When the follicular wall ruptures and the cumulus-oocyte complex is freed, ovulation occurs [78]. The residual tissue complex is converted into the luteal phase, a solid tissue mass. The sex steroid progesterone is synthesized and released

largely by the luteal phase [79]. The luteal phase is the final stage of the menstrual cycle, and in this stage that the secretion of progesterone and estradiol rises and falls significantly. Regression of the luteal phase is linked to a decrease in hormone output. At the so-called luteal-follicular transition, the concentration of FSH begins to grow in late luteal phase [80].

1.3. 3 Estrogen

Estrogen along with progesterone are the major hormones released by the ovaries [81]. Estrogen regulates physiological and pathological processes in the reproductive, cardiovascular, skeletal, endocrine, neurological, and immunological systems in both women and men, mediating a variety of effects across the body. As a result, it is involved in a variety of disorders, including different malignancie infertility, endometriosis, polycystic ovary syndrome [82]. The main effects of estrogen are the promotion of female secondary sex characteristics and the preparation of the uterus for ovulation and conception, It also has vascular benefits, such as increasing blood flow and forming new blood vessels, as well as endometrial and breast growth-promoting properties [83]. The liver is the primary site of estrogen metabolic breakdown, and it slowly absorbs estrogens over time, transforming them to a soluble form that may be expelled in the bile [84].

Most portions of the female reproductive system change when estrogen levels drop and menopause approaches, which usually occurs at the age of fifty [85]. Estrogens are traditionally thought of as feminine hormones, yet their importance in male reproduction cannot be overstated, as evidenced by current research [86, 87]. Furthermore, estrogen receptors have been identified in males from infancy to adulthood [88]. The pituitary is stimulated by the high estrogen level to create high levels

of LH and FSH all of a sudden. The oocyte undergoes final maturational modifications because of the LH surge, which leads to ovulation [89].

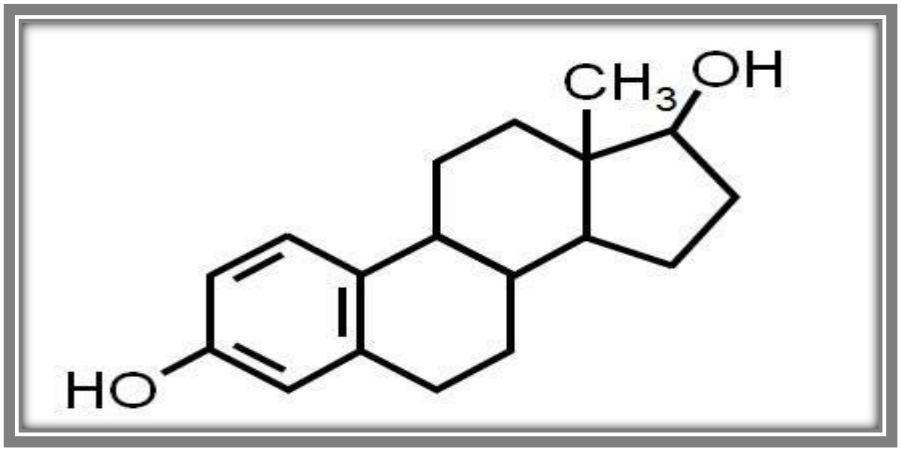

Figure 1.5 Estrogen hormone structure [90].

1.3. 4 Progesterone

The hormone progesterone is a steroid hormone. This sex hormone is required for a successful pregnancy. The chemical signal employed by the ovary to change the endometrial lining of the uterus into a highly secretory tissue that can maintain the fertilized egg is progesterone, which is released by cells of the luteal phase in a non-pregnant woman [91]. Progesterone is produced in the ovaries, adrenal glands, and the placenta during pregnancy. It is also kept in the adipose tissue. When it comes to women, during the pre - ovulatory phase of the menstrual cycle, progesterone levels are low. After ovulation, they rise and remain elevated during the luteal phase. Before ovulation, progesterone levels are normally less than 2ng/ml, and after ovulation, they are greater than 5ng/ml, about 7 days before menstruation [92].

The oocytes is released from the follicle, surrounded by cumulus cells during ovulation. After ovulation, the luteal phase begins and lasts 14 days in most women with little variance, a luteal phase is formed when the remaining follicular cells in the ovary are luteinized and

generate progesterone. LH secretion is still continuing on, activity ensures a steady supply of progesterone, which keeps the endometrium in good shape, pregnant woman's preparation, during the luteal phase of the cycle, progesterone levels are at their greatest. The high levels of progesterone also restrict FSH and LH secretion to the point where no more is produced ovarian follicular growth during that cycle [93].

Progesterone is necessary before and during pregnancy because it helps to maintains the endometrium and so the remain pregnancy [94].Women's endogenous sex hormones (estrogen and progesterone) fluctuate in a cyclic pattern from menstruation to menopause, resulting in the physiological process known as the menstrual cycle. This cycle is highly influenced by a pituitary feedback system the hypothalamus and hormones. The hormones estrogen and progesterone are principally responsible for the menstrual. Various parts of the menstrual cycle are regulated, with the postmenstrual phase being the most important. The menstrual cycle is estrogen-dependent, while the premenstrual phase is progesterone-dependent [95].

Figure 1.6 Progesterone hormone structure [96].

1.3. 5 Prolactin

Prolactin (PRL) is a protein produced by the PRL gene in humans [(97)]. The pituitary, myometrium, breast, lymphocytes, leucocytes, and prostate all make prolactin in humans [(98)]. Endocrine cells of the hypothalamus regulate pituitary prolactin secretion, the most significant of which are the central nervous system, which produce dopamine (prolactin inhibitory hormone) to function as a regulator [(99)]. Prolactin induces the mammary glands to make milk (lactation); high levels of prolactin in the blood during pregnancy lead the mammary glands to expand and prepare for milk production. When progesterone levels fall by the end of pregnancy and a suckling trigger is present, milk production begins. Prolactin levels also differ depending on factors such as age, gender, menstrual cycle stage, and pregnancy. Before a prolactin value may be accurately interpreted, the circumstances surrounding the test (assay, patient state, etc.) must be considered [(100)]. Hyperprolactinemia is a hormonal imbalance that affects women of reproductive age. It is very common in women who are experiencing reproductive or menstrual problems [(101)]. Hypogonadism in women that have hyperprolactinemia is thought to be caused by high circulating levels of prolactin interfering with gonadotropin action at the ovarian level, as well as reduced gonadal steroid secretion, which affects positive feedback effects at the ovarian level. Levels of the hypothalamus and pituitary Infertility result from a lack of gonadotropin (FSH and LH) [(102)].

Prolactin hormone has a molecular weight of 23.000 Dalton and is made up of 199 amino acids [(103)]. It is involved in reproduction, calcium metabolism and behavior, among other things [(104)]. PRL has predominantly been discovered as a primary lactation-stimulating agent in the postpartum phase. However, aside from its historical significance, other hormones are affected by this hormone's actions. Characteristics of

homeostasis in humans, such as osmoregulation and metabolism and immunological and endocrine systems [105]. The most common signs and symptoms of a chronic increase in prolactin levels in the blood include Breast pathology, sexual dysfunction, and reproductive dysfunction, anomalies linked to behavioral and emotional changes, immunologic issues and depression [106].

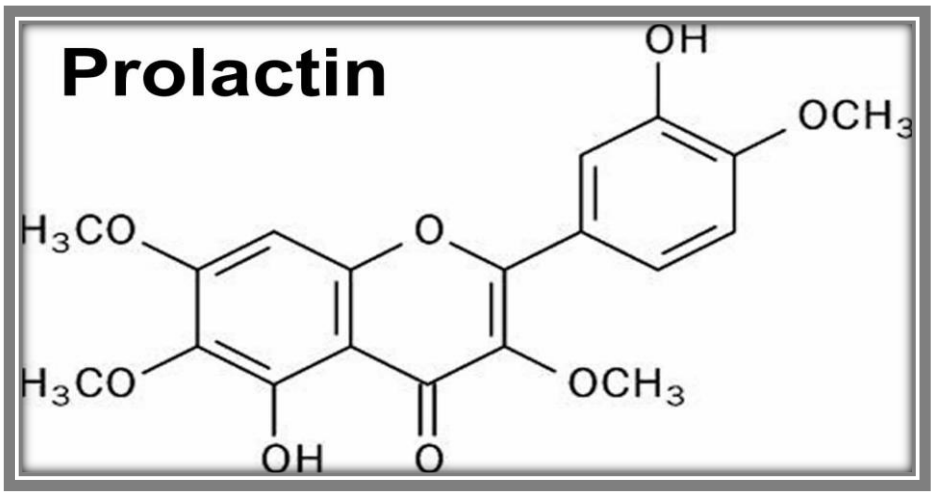

Figure 1.7 Prolactin hormone structure [107].

1.3. 6 Testosterone

Although testosterone is commonly associated with men, women in the ovaries and adrenal glands also generate it. Women may have symptoms of what is now known as androgen deficiency when testosterone levels fall with age and menopause. Some experts call it "androgen insufficiency syndrome," and it includes the typical symptoms of menopause [108].Thirty to fifty percent of androgen production takes place in peripheral tissues like muscle, bone, and fat [109]. Reduced libido and other menopause-related symptoms may occur in women who are experiencing a testosterone deficiency as a result of natural or surgically induced menopause [110]. In both males and females, testosterone plays an important role in health and well-being [111]. Testosterone like other

steroid hormones, is made from cholesterol. Around half of the testosterone hormone in circulation in normal women comes from peripheral conversion to a hormone [112]. Testosterone is made up of 17beta-hydroxy [113].

In women, testosterone has a biological role in follicular atresia and it is present in the lowest concentration in the early stages. The luteal phase of the cycle begins with the follicular phase and progresses to the mid-cycle peak. Concentrations are higher in the late follicular phase than they are in the early follicular phase [114]. The main aberrant hormonal aspect of polycystic ovary syndrome is excessive ovarian androgen production [115]. Testosterone has various functions as a hormone, such as secondary sexual characteristics; nevertheless, due of its effects on metabolic pathways, it has an impact on the cardiovascular, neuromuscular, and central neurological systems [116]. The testes in men and the ovaries in women are the primary producers of testosterone. Men's testosterone levels are typically 7-8 times higher than women's [117].

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are anterior pituitary hormones that play a part in the complex control of testosterone synthesis and release [118]. Obesity has been related to altered levels of reproductive hormones, such as the sex hormone testosterone, in both men and women, resulting in infertility [119]. The level of testosterone hormone in females is usually low, and this amount is mostly from the process of converting endogenous weak androgen steroid hormone, which is produced by the ovary, and adrenalin to testosterone, and getting this transfiguration outside the ovarian tissue, and notes that the male hormone in females has two images: a small part

of it is free, the active part of this hormone, and the largest part is found in combination with the compound, the inactive part of this hormone [120].

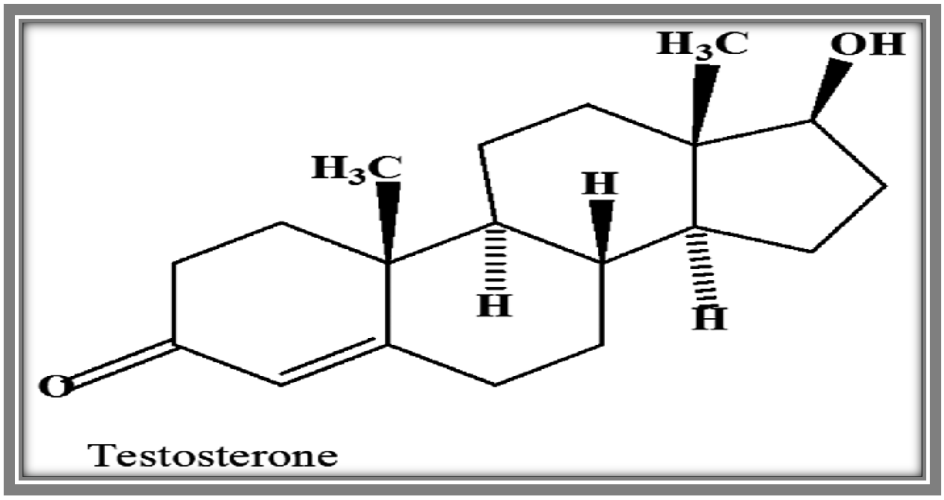

Figure 1.8 Testosterone hormone structure [121].

1.3. 7 INHIBIN B

The gonads secrete two essential hormones that are associated with FSH and LH. INHBB and Testosterone, to be precise. It is a glycoprotein hormone with one α-subunit and β -subunit. It has a molecular weight of 32 k Dalton. In most cases, unbound subunits have no physiological impact. INHBB is a gonadal dimer polypeptide hormone that regulates the synthesis and secretion of follicle stimulating hormone (FSH) in a negative feedback loop. Its bioactivity is thus dependent on the creation of a dimer structure. A hormone regulates the hypothalamic-pituitary-gonadal axis. Throughout pubertal development and childhood [122]. INHBB levels are a better indicator of infertility result of recurrent miscarriages than FSH and LH. It is concentrations in infertile patients may provide significant information on spermatogenesis and may act as a more direct spermatogenesis measure than FSH [123]. INHBB serves a physiological role in reproductive endocrinology applications in both men and females, according to decades of research.

In a woman, it is regarded as a more advanced marker. As it is closely related to the number of ovarian follicles, it is sensitive for ovarian follicle number [124].

INHBB has a direct negative feedback effect on the pituitary gland when levels are high in the blood, resulting in a reduction in FSH. As a result, a higher level of serum INHBB in reproductive-age women is one of the key determinants in maintaining a low serum FSH level. However, as they become older, the quality and quantity of their ovarian follicles decline, the level of serum INHBB declines gradually, and the inhibitory impact on FSH is diminished, which is one of the major causes for their serum FSH levels steadily rising [125,126]. Its levels are typically at their maximum during the follicular phase of the menstrual cycle and lowest during the luteal phase, as evidenced by recent research in normal ovulatory women, and its concentration rises during the luteal follicular transition. The mid-follicular phase is when INHBB levels are highest, while the late follicular phase is when they are lowest [127].

When women experience weight gain, anorexia nervosa, and an increase in adipose tissue their INHBB levels rise [128]. Researchers gradually understood the role of INHBB in female fertility after years of research. Previous research revealed that INHBB could be used to monitor ovarian aging, diagnose premature ovarian failure (POF) or premature ovarian insufficiency (POI), assess ovarian function in cancer survivors, discovered that a decrease in INHBB was the earliest sign of follicle number loss as women aged [129].

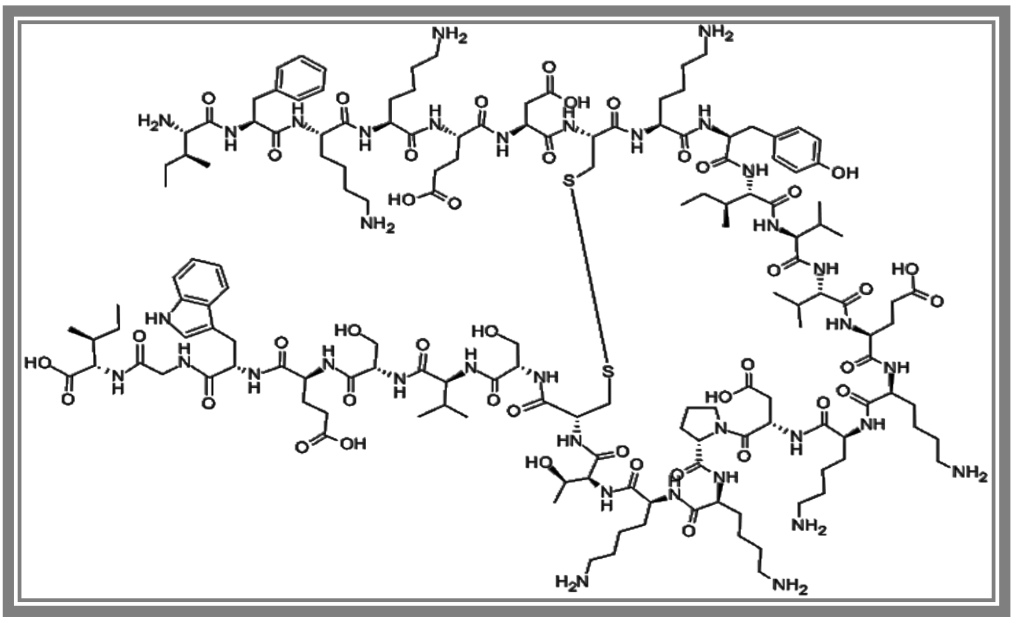

Figure 1.9 INHIBIN B hormone structure [130].

1.3. 8 ACTIVIN A

They are gonadal proteins that stimulate the production and release of pituitary follicle-stimulating hormone (FSH) [131]. It is discovered to be beneficial in a number of studies, not just in the ovaries and testes, but also in other parts of the body [132]. The placenta is the primary source of ACTIVIN A in the maternal circulation throughout pregnancy [133]. ACTIVIN A levels in the mother's blood from approximately mid-pregnancy until near to term, there is an increase [134]. Falling fast after born [135]. Placental malfunction, as shown by intrauterine fetal development limitation, complicates pregnancies [136]. ACTIVIN A levels in the mother's blood are much greater than in a normal pregnancy. While higher placental production is assumed to be the source of these elevated ACTIVIN A levels in the blood [137]. The use of ACTIVIN A as a predictor of pregnancy failure has sparked considerable controversy among researchers, with some claiming that ACTIVIN A readings can identify pregnant women at danger of a missed abortion [138]. In Preeclampsia, the level of serum ACTIVIN A rises [139].

Due to unknown dates of last menstrual period or irregular cycles, clinical differential diagnosis with ultrasonography at 6–8 weeks of pregnancy is extremely difficult. The amount of ACTIVIN A stimulatory action is stronger throughout the same time span [140].

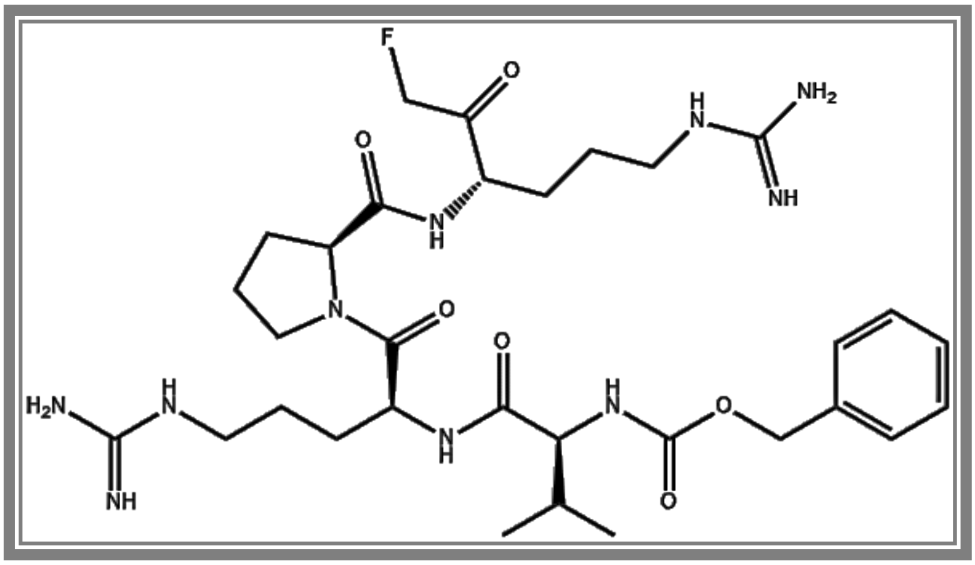

Figure 1.10 ACTIVIN A hormone structure [141].

ACTIVIN A is a 25 K Dalton homo-dimer that has been connected to a variety of biological processes, including mesoderm, neural cell, red blood cell, and follicular cell development as well as FSH release [142]. Normal bone marrow cells, as well as a variety of myeloid cell lines, produce ACTIVIN A [143]. ACTIVIN A signaling is involved in another of biological processes, such as muscle balance, inflammation, and wound healing [144].

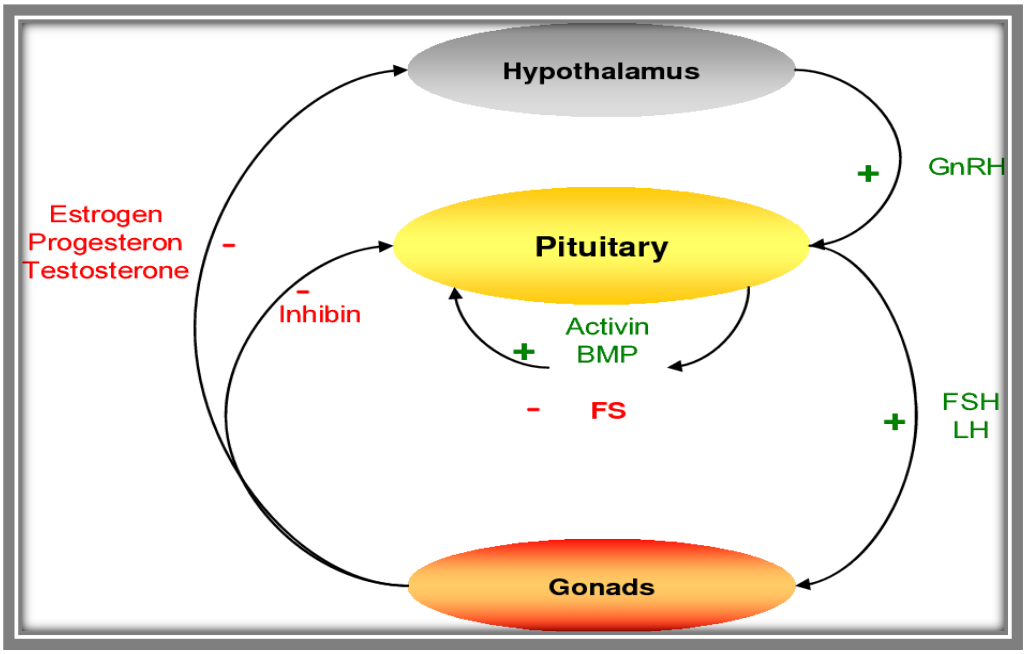

Figure 1.11 Hypothalamus-Pituitary-Gonads axis [145].

1.4 Aims of this study

The following aims will be provided by this study:

1. A study of the levels of a group of hormones in women who suffer from recurrent miscarriage to be used in predicting miscarriage.
2. To better understand what causes of recurrent miscarriages.
3. Finding the relationship between PCOS and the accompanying imbalance in the levels of some hormones and the occurrence of recurrent miscarriage.
4. Measuring the levels of the hormones INHIBIN B and ACTIVIN A, that is, as new variables, and the extent to which the levels of these variables differ with what is found in healthy women compared to women who suffer from recurrent miscarriage, and their adoption as indicators of miscarriage.

CHAPTER TWO

MATERIALS AND METHODS

2- Materials and Methods

2.1 Subjects and study design

A case-control study has been conducted at Gynecological and Obstetric Teaching Hospital in Kerbala city and the outpatient clinics for women. All samples were collected from October 2021 until September 2022. The study was conducted on 90 Iraqi women (non-pregnant) women's their age ranged (15-45) years within reproductive age. Group1 consisted of 40 women who had at least one child and had no miscarriage, They were free from signs and symptoms of diseases, their ages were matched with the age of recurrent miscarriage women. Group 2 of 50 women who had at least two recurrent miscarriages and who had polycystic ovary syndrome. Women who received a medical examination and a short questionnaire that asked about the woman's age, weight, height, number of miscarriages, duration of miscarriage, date of last miscarriage, number of births, regular or irregular of menses, addition to other diseases such as(diabetes mellitus, hypertension, heart disease, kidney disease, ectopic pregnancy, thyroid gland disease, smoking, drugs). All information from women was listed in table (2.1).

Table 2.1 Questionnaire of this study

Patient profile	Control profile
Age	Age
Date	Date
Address	Address
Weight	Weight
Length	Length
No. of births	No. of births
No. of day of menstrual cycle	No. of day of menstrual cycle
Regular or irregular menses	Regular or irregular menses
No. of miscarriages	Other disease
Duration of miscarriage	Diabetes mellitus
Date of last miscarriage	Hypertension
Other disease	Heart disease
Diabetes mellitus	Kidney disease
Hypertension	Ectopic pregnancy
Heart disease	Thyroid gland disease
kidney disease	Smoking
Ectopic pregnancy	Drugs
Thyroid gland disease	
Smoking	
Drugs	

2.2 Groups of this study

In two study groups, this was confirmed:

1. Controls group (healthy): The healthy group include 40 non- pregnant women who were able to have children, their ages range between (15-45) years.

2. Patients group: The group consists of 50 non-pregnant women suffering from recurrent miscarriage because of PCOS, their ages range between (15-45) years.

All of the female gave verbal informed permission, and Kerbala University approved the research procedure.

2.3 Collection Blood Samples

The Blood samples were collection from non- pregnant women (Gynecological and Obstetric Teaching Hospital and outpatient clinics) in order to do hormones testing (LH, FSH, prolactin, progesterone, testosterone, ESTROGEN, INHIBIN B, ACTIVIN A). A 5 ml medical syringe was used to draw 5 ml of blood, which was then put into gelatine tubes (also known as gel tubes) devoid of anti-clotting substances since they contain a gelatinous ingredient that helps in the separation of serum following centrifugation. The samples were left to stand at room temperature for 15 minutes before being centrifuged for 10 minutes at a speed of 2500 rounds per minute to separate the serum, which was kept at -20º C unless it was needed right away.

2.4 Exclusion criteria

Women suffering from diabetes mellitus, hypertension, heart disease, kidney disease, thyroid gland diseases, ectopic pregnancy, other than PCOS, obesity effect and maternal age.

2.5 The chemicals

The chemicals used in this study are described in table (2.2).

Table 2.2 The chemicals

Chemicals	Company and Origin
LH ELISA Kit	China
FSH ELISA Kit	China
Estrogens ELISA Kit	China
Progesterone ELISA Kit	China
Prolactin ELISA Kit	China
Testosterone ELISA Kit	China
INHIBIN B ELISA Kit	USA
ACTIVIN A ELISA Kit	USA

2.6 Instrument analysis and equipment

Equipment and instruments that are used in the this study are shown in table (2.3).

Table 2.3 Instrument analysis and equipment

Instruments	Supplied Company
CL-900i	Mindray-China
Absorbance ELISA microplate reader	BioTek-USA
ELISA microplate washer	BioTek-USA
Centrifuge	Heraeus-Germany
Refrigerator	Kiriazi-Egypt
Gelatin tubes (Jell tube)	Germany
Eppendorf tips 5ml	China

2.7 Measurement of body mass index (BMI)

Body mass index were calculated from the following equation [146].

BMI = Weight (Kg) / Height (m^2)

They were classified into three groups:

1. The BMI of normal weight range is (18.5-24.9) Kg/m^2
2. The BMI of overweight range is (25-29.9) Kg/m^2
3. The BMI of obese is ≥ 30 Kg/m^2

2.8 Fertility hormones tests

2.8.1 Measuring serum LH levels of hormone

LH levels were measured in blood serum using the CL-900i device by Mindray Company in China with the use of a ready-made test kit, following the instructions provided with the kit.

Principle of a test

LH assay is a sandwich chemiluminescence immunoassay. The sample (or calibrator/control, if applicable), magnetic microbeads coated

with anti-LH monoclonal antibody, ABEI labelled with another monoclonal antibody are mixed thoroughly and incubated at 37° C, forming sandwich complexes. After precipitation in a magnetic field, the supernatant is decanted and then perform a wash cycle. Subsequently, the starter 1+2 are added to initiate a chemiluminescent reaction. The light signal is measured by a photomultiplier within 3 seconds as relative light units (RLUs), which is proportional to the concentration of LH present in the sample (or calibrator/control, if applicable).

Procedure

1. The micro plate wells were formatted for each serum reference, control and patient specimen to be assayed in duplicate.
2. A volume of 50 µl of appropriate serum reference, control or specimen was pipetted into the assigned well.
3. A volume of 100 µl of LH-enzyme reagent was added to all wells.
4. The micro plate was gently swirled for 20-30 seconds to mix and cover.
5. The plate was incubated for 60 minutes at room temperature.
6. The contents of the micro plate were discarded by decantation or aspiration, if decanting, blot the plate dry with absorbent paper.
7. The plate was washed three times with 300 µl of the wash buffer.
8. A volume of 100 µl of working substrate solution was added to all wells, reagents were always added in the same order to minimize reaction time differences between wells, the plate wasn´t shacked after substrate addition.
9. The plate was incubated at room temperature for 15 minutes. A volume of 10-50 µl of stop solution was added to each well and was gently mixed for 15-20 seconds.

10. The absorbance was read in each well at 450 nm in a micro plate reader. The results should be read within 30 minutes of adding the stop solution.

Calculations

The standard curve of LH determination was plotted in Figure (2.2) and the LH level in each sample was determined.

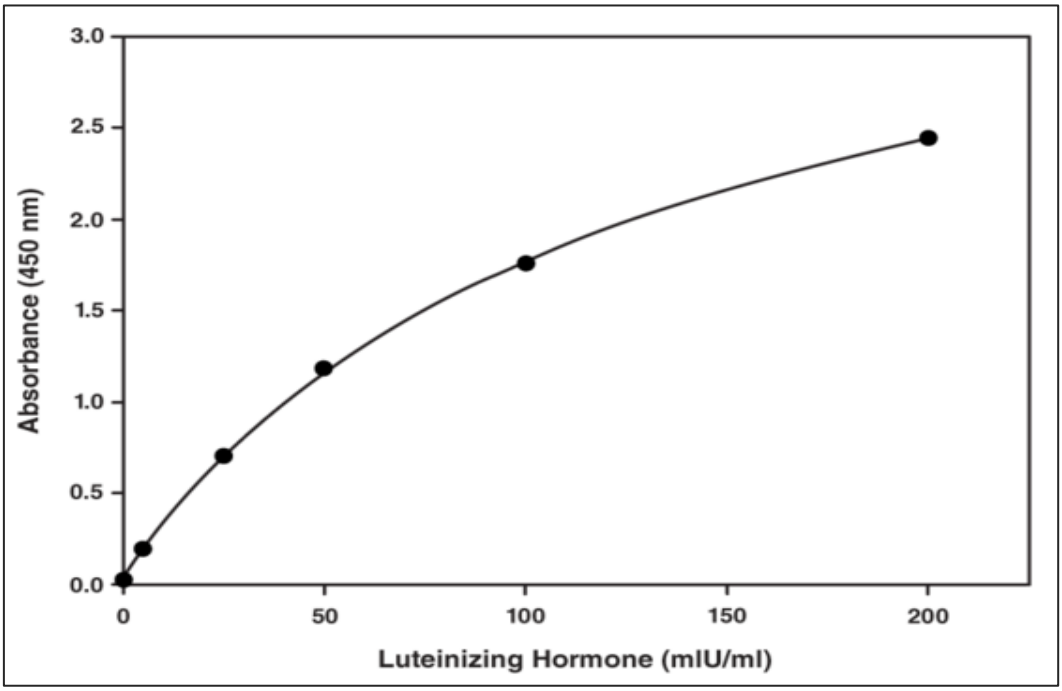

Figure 2.2 Standard curve of determination of LH concentration.

2.8.2 Measuring serum FSH levels of hormone

FSH levels were measured in blood serum using the CL-900i device by Mindray Company in China with the use of a ready-made test kit, following the instructions provided with the kit.

Principle of a test

FSH assay is a sandwich chemiluminescence immunoassay. The sample (or calibrator/control, if applicable), magnetic microbeads coated with anti-FSH monoclonal antibody, ABEI labelled with another monoclonal antibody are mixed thoroughly and incubated at 37° C,

forming sandwich complexes. After precipitation in a magnetic field, the supernatant is decanted and then perform a wash cycle. Subsequently, the starter 1+2 are added to initiate a chemiluminescent reaction. The light signal is measured by a photomultiplier within 3 seconds as relative light units (RLUs), which is proportional to the concentration of FSH present in the sample (or calibrator/control, if applicable).

Procedure

1. The micro plate wells were formatted for each serum reference, control and patient specimen to be assayed in duplicate.
2. A volume of 50 µl of appropriate serum reference, control or specimen was pipetted into the assigned well.
3. A volume of 100 µl of FSH-enzyme reagent was added to all wells.
4. The micro plate was gently swirled for 20-30 seconds to mix and cover.
5. The plate was incubated for 60 minutes at room temperature.
6. The contents of the micro plate were discarded by decantation or aspiration, if decanting, blot the plate dry with absorbent paper.
7. The plate was washed three times with 300 µl of the wash buffer.
8. A volume of 100 µl of working substrate solution was added to all wells, reagents were always added in the same order to minimize reaction time differences between wells, the plate wasn´t shacked after substrate addition.
9. The plate was incubated at room temperature for 15 minutes. A volume of 10-50 µl of stop solution was added to each well and was gently mixed for 15-20 seconds.
10. The absorbance was read in each well at 450 nm in a micro plate reader. The results should be read within 30 minutes of adding the stop solution.

Calculations

The standard curve of FSH determination was plotted in Figure (2.3) and the FSH level in each sample was determined.

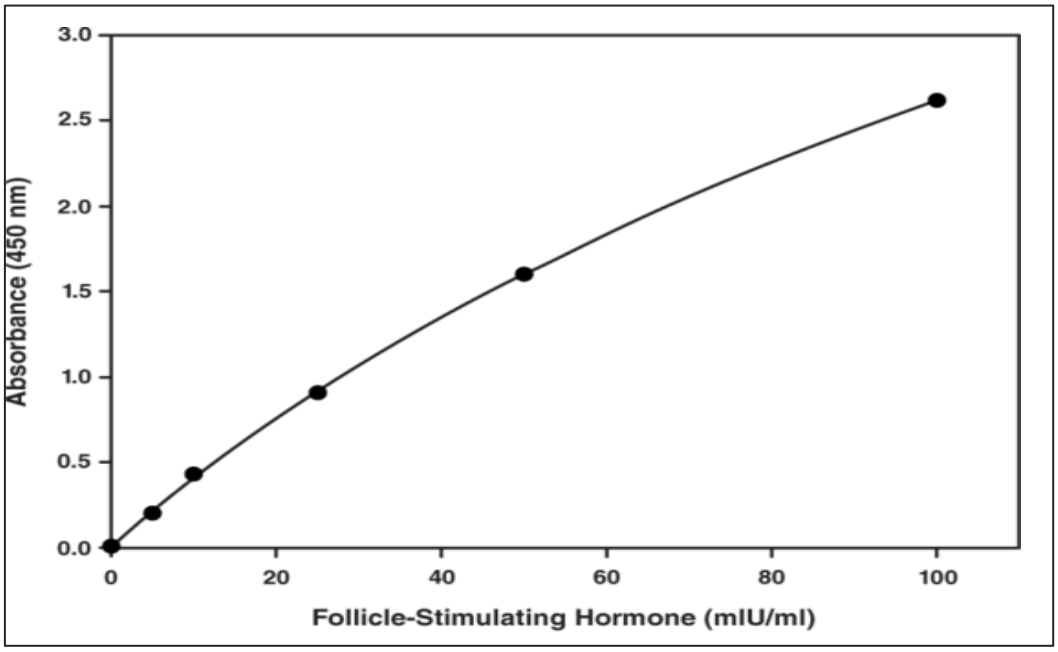

Figure 2.3 Standard curve of determination of FSH concentration.

2.8.3 Measuring serum ESTROGEN levels of hormone

ESTROGEN levels were measured in blood serum using the CL-900i device by Mindray Company in China with the use of a ready-made test kit, following the instructions provided with the kit.

Principle of a test

ESTROGEN assay is a sandwich chemiluminescence immunoassay. The sample (or calibrator/control, if applicable), magnetic microbeads coated with anti-ESTROGEN monoclonal antibody, ABEI labelled with another monoclonal antibody are mixed thoroughly and incubated at 37° C, forming sandwich complexes. After precipitation in a magnetic field, the supernatant is decanted and then perform a wash cycle. Subsequently, the starter 1+2 are added to initiate a

chemiluminescent reaction. The light signal is measured by a photomultiplier within 3 seconds as relative light units (RLUs), which is proportional to the concentration of ESTROGEN present in the sample (or calibrator/control, if applicable).

Procedure

1. The micro plate wells were formatted for each serum reference, control and patient specimen to be assayed in duplicate.
2. A volume of 50 µl of appropriate serum reference, control or specimen was pipetted into the assigned well.
3. A volume of 100 µl of ESTROGEN-enzyme reagent was added to all wells.
4. The micro plate was gently swirled for 20-30 seconds to mix and cover.
5. The plate was incubated for 60 minutes at room temperature.
6. The contents of the micro plate were discarded by decantation or aspiration, if decanting, blot the plate dry with absorbent paper.
7. The plate was washed three times with 300 µl of the wash buffer.
8. A volume of 100 µl of working substrate solution was added to all wells, reagents were always added in the same order to minimize reaction time differences between wells, the plate wasn´t shacked after substrate addition.
9. The plate was incubated at room temperature for 15 minutes. A volume of 10-50 µl of stop solution was added to each well and was gently mixed for 15-20 seconds.
10. The absorbance was read in each well at 450 nm in a micro plate reader. The results should be read within 30 minutes of adding the stop solution.

Calculations

The standard curve of ESTROGEN determination was plotted in Figure (2.4) and the ESTROGEN level in each sample was determined.

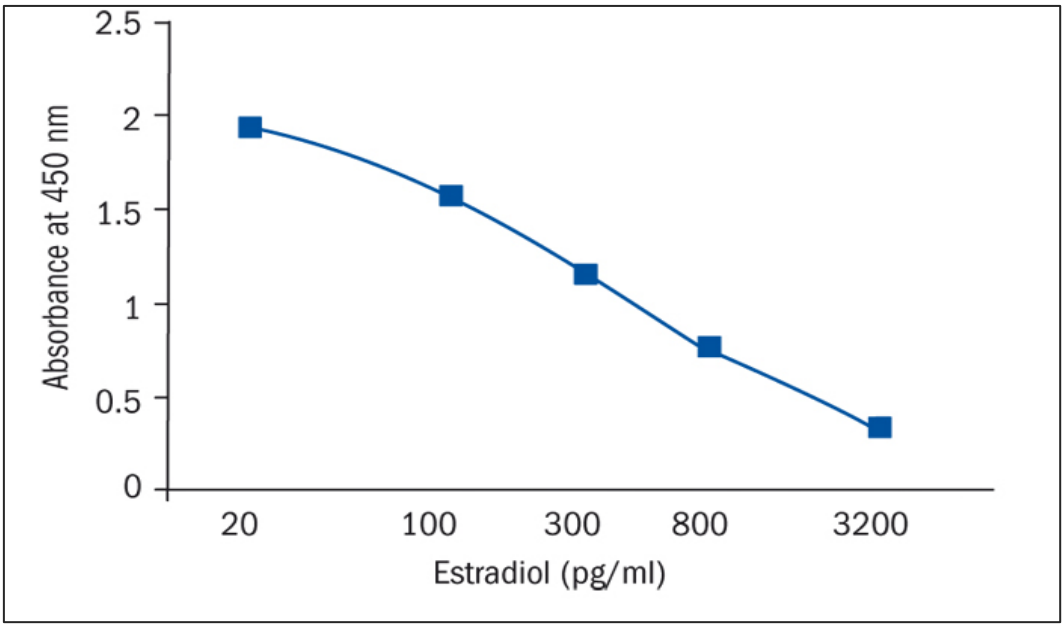

Figure 2.4 Standard curve of determination of ESTROGEN concentration.

2.8.4 Measuring serum progesterone levels of hormone

Progesterone levels were measured in blood serum using the CL-900i device by Mindray Company in China with the use of a ready-made test kit, following the instructions provided with the kit.

Principle of a test

Progesterone assay is a sandwich chemiluminescence immunoassay. The sample (or calibrator/control, if applicable), magnetic microbeads coated with anti-progesterone monoclonal antibody, ABEI labelled with another monoclonal antibody are mixed thoroughly and incubated at 37° C, forming sandwich complexes. After precipitation in a magnetic field, the supernatant is decanted and then perform a wash cycle. Subsequently, the starter 1+2 are added to initiate a chemiluminescent reaction. The light signal is measured by a

photomultiplier within 3 seconds as relative light units (RLUs), which is proportional to the concentration of progesterone present in the sample (or calibrator/control, if applicable).

Procedure

1. The micro plate wells were formatted for each serum reference, control and patient specimen to be assayed in duplicate.
2. A volume of 50 µl of appropriate serum reference, control or specimen was pipetted into the assigned well.
3. A volume of 100 µl of progesterone-enzyme reagent was added to all wells.
4. The micro plate was gently swirled for 20-30 seconds to mix and cover.
5. The plate was incubated for 60 minutes at room temperature.
6. The contents of the micro plate were discarded by decantation or aspiration, if decanting, blot the plate dry with absorbent paper.
7. The plate was washed three times with 300 µl of the wash buffer.
8. A volume of 100 µl of working substrate solution was added to all wells, reagents were always added in the same order to minimize reaction time differences between wells, the plate wasn´t shacked after substrate addition.
9. The plate was incubated at room temperature for 15 minutes. A volume of 10-50 µl of stop solution was added to each well and was gently mixed for 15-20 seconds.
10. The absorbance was read in each well at 450 nm in a micro plate reader. The results should be read within 30 minutes of adding the stop solution.

Calculations

The standard curve of progesterone determination was plotted in Figure (2.5) and the progesterone level in each sample was determined.

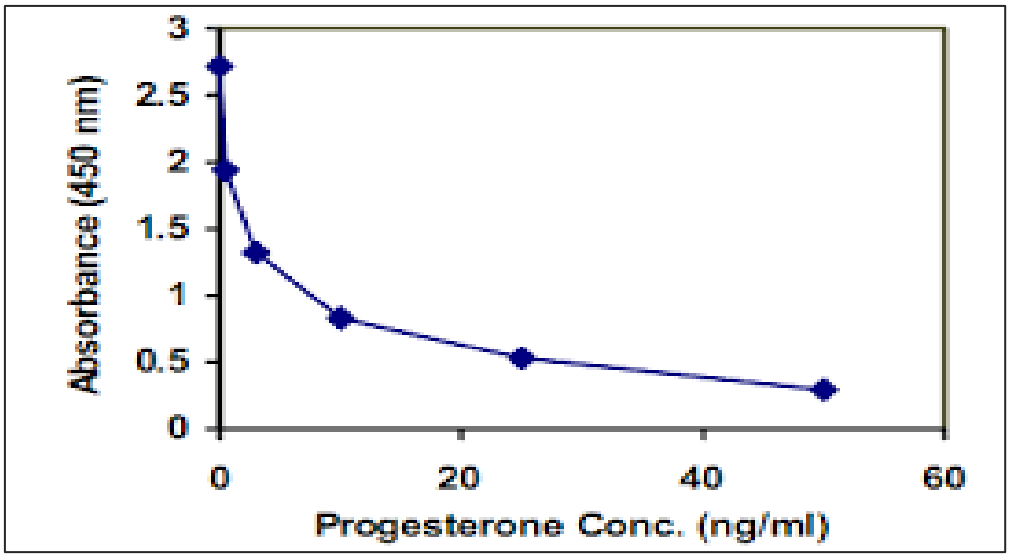

Figure 2.5 Standard curve of determination of progesterone concentration.

2.8.5 Measuring serum prolactin levels of hormone

Prolactin levels were measured in blood serum using the CL-900i device by Mindray Company in China with the use of a ready-made test kit, following the instructions provided with the kit.

Principle of a test

Prolactin assay is a sandwich chemiluminescence immunoassay. The sample (or calibrator/control, if applicable), magnetic microbeads coated with anti-PRL monoclonal antibody, ABEI labelled with another monoclonal antibody are mixed thoroughly and incubated at 37° C, forming sandwich complexes. After precipitation in a magnetic field, the supernatant is decanted and then perform a wash cycle. Subsequently, the starter 1+2 are added to initiate a chemiluminescent reaction. The light signal is measured by a photomultiplier within 3 seconds as relative light

units (RLUs), which is proportional to the concentration of prolactin present in the sample (or calibrator/control, if applicable).

Procedure

1. The micro plate wells were formatted for each serum reference, control and patient specimen to be assayed in duplicate.
2. A volume of 50 µl of appropriate serum reference, control or specimen was pipetted into the assigned well.
3. A volume of 100 µl of prolactin-enzyme reagent was added to all wells.
4. The micro plate was gently swirled for 20-30 seconds to mix and cover.
5. The plate was incubated for 60 minutes at room temperature.
6. The contents of the micro plate were discarded by decantation or aspiration, if decanting, blot the plate dry with absorbent paper.
7. The plate was washed three times with 300 µl of the wash buffer.
8. A volume of 100 µl of working substrate solution was added to all wells, reagents were always added in the same order to minimize reaction time differences between wells, the plate wasn´t shacked after substrate addition.
9. The plate was incubated at room temperature for 15 minutes. A volume of 10-50 µl of stop solution was added to each well and was gently mixed for 15-2 seconds.
10. The absorbance was read in each well at 450 nm in a micro plate reader. The results should be read within 30 minutes of adding the stop solution.

Calculations

The standard curve of prolactin determination was plotted in Figure (2.6) and the prolactin level in each sample was determined.

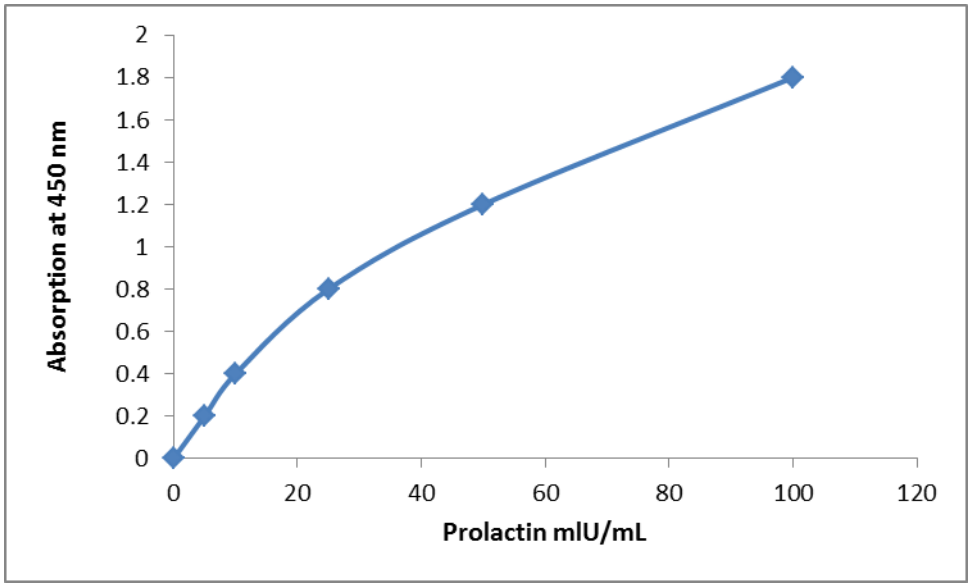

Figure 2.6 Standard curve of determination of prolactin concentration.

2.8.6 Measuring serum testosterone levels of hormone

Testosterone levels were measured in blood serum using the CL-900i device by Mindray Company in China with the use of a ready-made test kit, following the instructions provided with the kit.

Principle of a test

Testosterone assay is a sandwich chemiluminescence immunoassay. The sample (or calibrator/control, if applicable), magnetic microbeads coated with anti-testosterone monoclonal antibody, ABEI labelled with another monoclonal antibody are mixed thoroughly and incubated at 37° C, forming sandwich complexes. After precipitation in a magnetic field, the supernatant is decanted and then perform a wash cycle. Subsequently, the starter 1+2 are added to initiate a chemiluminescent reaction. The light signal is measured by a photomultiplier within 3 seconds as relative light units (RLUs), which is proportional to the concentration of testosterone present in the sample (or calibrator/control, if applicable).

Procedure

1. The micro plate wells were formatted for each serum reference, control and patient specimen to be assayed in duplicate.
2. A volume of 50 µl of appropriate serum reference, control or specimen was pipetted into the assigned well.
3. A volume of 100 µl of testosterone-enzyme reagent was added to all wells.
4. The micro plate was gently swirled for 20-30 seconds to mix and cover.
5. The plate was incubated for 60 minutes at room temperature.
6. The contents of the micro plate were discarded by decantation or aspiration, if decanting, blot the plate dry with absorbent paper.
7. The plate was washed three times with 300 µl of the wash buffer.
8. A volume of 100 µl of working substrate solution was added to all wells, reagents were always added in the same order to minimize reaction time differences between wells, the plate wasn´t shacked after substrate addition.
9. The plate was incubated at room temperature for 15 minutes. A volume of 10-50 µl of stop solution was added to each well and was gently mixed for 15-20 seconds.
10. The absorbance was read in each well at 450 nm in a micro plate reader. The results should be read within 30 minutes of adding the stop solution.

Calculations

The standard curve of Testosterone determination was plotted in Figure (2.7) and the testosterone level in each sample was determined.

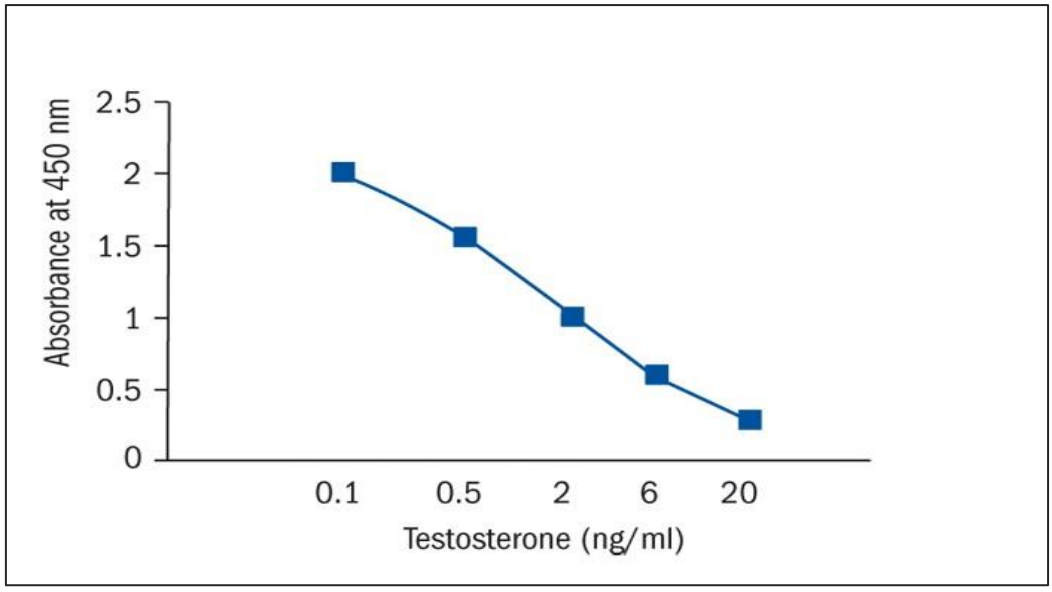

Figure 2.7 Standard curve of determination of testosterone concentration.

2.8.7 Measuring serum INHIBIN B levels of hormone

INHIBIN B level was determined by the method of the kit of RayBio® USA with the use of a ready-made test kit, following the instructions provided with the kit.

Principle of a test

The RayBio® INHIBIN B enzyme immunoassay (EIA) kit is an in vitro quantitative assay for detecting INHIBIN B peptide based on the principle of competitive enzyme immunoassay. The micro plate in the kit pre-coated with anti-INHIBIN B secondary antibody. After a blocking step and incubation of the plate with anti-INHIBIN B antibody, both biotinylated INHIBIN B peptide and peptide standard or targeted peptide in samples interacts competitively with the INHIBIN B antibody. Uncompleted (bound) biotinylated INHIBIN B peptide then interacts with streptavidin-horseradish peroxidase (SA-HRP), which stimulate a colour development reaction. The intensity of colorimetric signal is directly proportional to the amount of biotinylated peptide (SA-HRP)

complex and inversely proportional to the amount of INHIBIN B peptide in the standard or samples. This is due to the competitive binding to INHIBIN B antibody between biotinylated INHIBIN B peptide and peptide in standard or samples.

Prepare the Reagent

1. Kit reagents were kept on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.
2. Diluent B (Item E) was diluted 5-fold with deionized or distilled water.
3. The anti-INHIBIN B antibody vial (Item N) was briefly centrifuged before use, a volume of 50 µl of 1X of diluent B was added into the vial to prepare a detection antibody concentrate . Solution was pipette up and down to mix gently.
4. The antibody concentrate was the diluted 100-fold with 1X of diluent B. This is anti-INHIBIN B antibody working solution, which will be used in step 2 of the assay procedure.
5. The vial of INHIBIN B (Item F) was briefly centrifuged before use, 5 µl was add to 5 ml of the appropriate assay diluent. Solution was pipette up and down to mix gently. The final concentration of INHIBIN B will be 100pg/ml. This solution will only be used as the diluent in step 6 of reagent preparation.
6. Preparation of standards: 6 micro-tubes were classified with the following concentrations:10000pg/ml, 1000pg/ml, 100pg/ml, 10pg/ml, 1pg/ml and 0pg/ml. 450 µl of INHIBIN B solution was pipette into each tube, except for the 10000pg/ml (leave this one empty). It is very important to make sure the concentration of INHIBIN B is 100pg/ml in all standards.

a. The vial of INHIBIN B (Item C) was briefly centrifuged, in the tube classified 10000pg/ml, a volume of 8 µl of item C was pipette and 792 µl

of 100pg/ml INHIBIN B solution (prepared in step 5). This is INHIBIN B stock solution (10000pg/ml INHIBIN B, 100pg/ml INHIBIN B), and it mixed thoroughly this solution serves as the first standard.

b. To make 1000pg/ml standard, 50 µl of INHIBIN B stock solution was pipette the tube classified 1000pg/ml, and it mix thoroughly.

c. This step was repeat with each successive concentration preparing a dilution series as shown in the illustration below. Each time volume 450 µl of INHIBIN B and 50 µl of the prior concentration were used until 1pg/ml is reached. And it mix each tube thoroughly before the next transfer.

d. The final tube (0pg/ml INHIBIN B, 100pg/ml INHIBIN B) serves as the zero standard (or total binding).

7. Volume of 10-fold dilution of (Item F) was prepared to do this 2 µl of Item F was added to 18 µl of the appropriate diluent. This solution will be used in steps 8 and 10.

8. Positive control preparation : the positive control vial (Item M) to the tube of the item M was briefly centrifuged, a volume of 101 µl 1X of diluent B was added. Also 2 µl of 10-fold diluted item F (prepared in step 7) was added to the tube. This is a 2-fold dilution of the positive control. It was mix thoroughly.

9. If (Item B) 20X wash concentrate contains visible crystals, it was warm to room temperature and it mixed gently until dissolved. A volume of 20 ml of wash buffer concentrate was diluted into deionized or distilled water to yield 400 ml of 1X wash buffer.

10. Sample preparation: diluent A+ INHIBIN B were used to dilute serum/plasma samples. For cell culture medium and other sample types, 1X of diluent B+ INHIBIN B were used as the diluent. It is very important to make sure the final concentration of the INHIBIN B is 100pg/ml in every sample.

11. HRP-Streptavidin vial (Item G) was briefly centrifuged before use. The HRP-Streptavidin concentrate was diluted 400-fold with 1X of diluent B.

Procedure

1. Kit of reagents were keep on ice during preparation steps. It is recommended that all standards and samples be run at least in duplicate.
2. A volume of 100 µl anti-INHIBIN B antibody (see reagent preparation step 4) was added to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycles/sec). It may also incubate overnight at 4 degrees C.
3. Solution was discard and it was washed wells 4 times with 1x wash buffer (200-300 µl each), washing may be done with a multichannel pipette or an automated plate washer. It was complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. A volume of 100 µl was added of each standard (see reagent preparation step 6), positive control (see reagent preparation step 8) and sample (see reagent preparation step 10) into appropriate wells. Be sure to include a blank well (diluent only). Cover wells and it was incubated for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) or overnight at Cº.
5. Solution discard and it washed 4 times as directed in step 3
6. A volume of 100 µl of prepared HRP-Streptavidin solution (see reagent preparation step 11) was added to each well. It was incubated for 45 minutes with gentle shaking at room temperature. It is recommended that incubation time should not be shorter or longer than 45 minutes.
7. Solution was discard wash 4 times as directed in step 3.

8. A volume of 100 µl of TMP was added one step substrate reagent (Item H) to each well. It was incubated for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).

9. A volume of 50 µl of stop solution (Item I) was added to each well. Read absorbance at 450 nm immediately.

Calculations

The standard curve of INHIBIN B determination was plotted in Figure (2.8) and the INHIBIN B level in each sample was determined. (B/Bo) was calculated from the following:

OD: Optical Density.

Percentage absorbance = (B blank OD) / (Bo-blank OD) where

B : OD of sample or standard and

Bo : OD of zero standard (total binding).

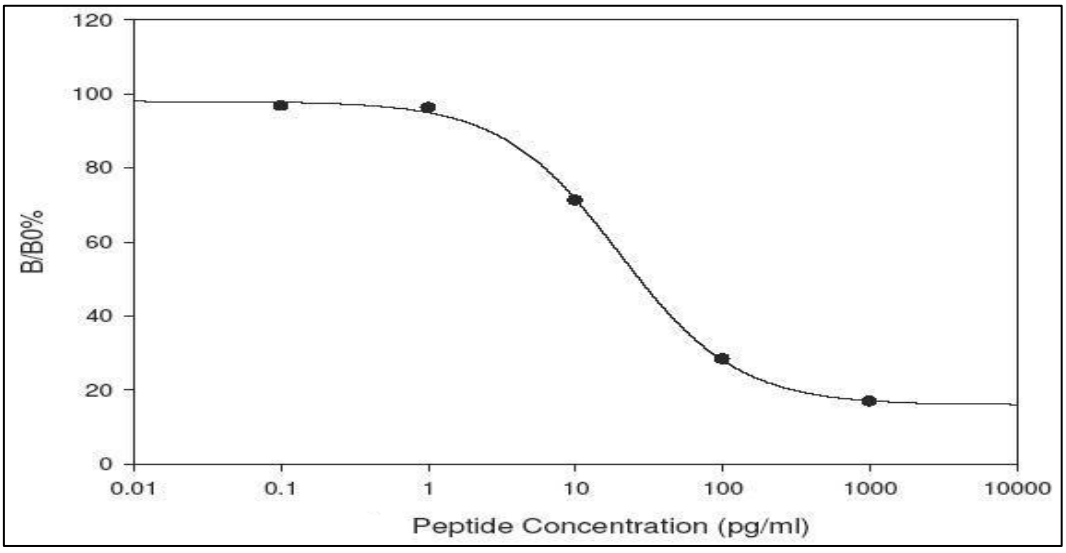

Figure 2.8 Standard curve of determination of INHIBIN B concentration.

2.8.8 Measuring serum ACTIVIN A levels of hormone

ACTIVIN A level was determined by the method of the kit of RayBio® USA with the use of a ready-made test kit, following the instructions provided with the kit.

Principle of a test

The RayBio® human ACTIVIN A ELISA (Enzyme-Linked Immunosorbent Assay) kit is an vitro enzyme-linked immunosorbent assay for the quantitative measurement of human ACTIVIN A in serum, plasma and urine. This assay employs an antibody specific for human ACTIVIN A coated on a 96-well plate. Standards and samples are pipetted into the well and ACTIVIN A present in a sample is bound to the well by the immobilized antibody. Wells are washed and biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. Wells are again washed, a TMB substrate solution is added to the wells and colour develops in proportion to the amount of ACTIVIN A bound. The stop solution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm.

Prepare the Reagent

1. All reagents and samples were brought to room temperature (18-25^{o} C) before use.

2. Sample dilution: if the samples need to be diluted, diluted A (Item D) was used for dilution of serum / plasma samples. 1X diluent B (Item E) was used for dilution of cell culture.

3. Preparation of standard: the vial of (Item C) was briefly spinned and then it was added 400 µl of diluent A in to (Item C) vial to prepare a 50ng/ml standard. The powder was dissolve thoroughly by a gentle mix. Volume of 50 µl ACTIVIN A standard (50ng/ml) from the vial of (Item C), was added into a tube with 450 µl of diluent A to prepare a 5000pg/ml standard solution. Volume of 300 µl of diluent A was pipetted into each

tube, the 5000pg/ml standard solution was used to produce a dilution series. It was mixed each tube thoroughly before the next transfer.

4. If the wash concentrate 20X (Item B) contains visible crystals, it was warm to room temperature and mixed until dissolved. Volume of 20 ml of wash buffer concentrate was diluted into deionized or distilled water to yield 400 ml of 1X wash buffer.

5. Detection antibody vial (Item F) was briefly spinned before use. Volume of 100 µl of 1X of diluent B was added into the vial to prepare a detection antibody concentrate. It pipetted up and down to mixed it gently (the concentrate can be stored at 4 Cº for 5 days). The detection antibody concentrate was diluted 80-fold with 1X of diluent B.

6. HRP-Streptavidin concentrate vial (Item G) was briefly spinned the and it was pipetted up and down to mix it gently before use. HRP-Streptavidin concentrate was diluted 200-fold with 1X of diluent B.

Procedure

1. All reagents and samples were brought to room temperature (18-25º C) before use. It is recommended that all standards and samples to be run at least in duplicate.

2. A volume of 100 µl of each standard (see reagent preparation step 2) and samples were added into appropriate wells. Wells were covered and incubate it for 2.5 hours at room temperature or overnight at room temperature or overnight at 4 Cº with gentle shaking.

3. Solution was discarded and wash it for 4 times with 1X wash solution, each well was washed by filling wash by filling with wash buffer (300 µl) using a multi-channel pipette or auto-washer. Complete removal of liquid at each step is essential to good performance. After the last wash,

remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. A volume 100 µl of 1X prepared biotinylated antibody (see reagent preparation step 6) was added to each well. It was incubated for 1 hour at room temperature with gentle shaking.

5. Solution was discarded. The wash was repeated as in step 3.

6. A volume of 100 µl of prepared streptavidin solution was added to each well. And incubate it for 45 minutes at room temperature with gentle shaking.

7. The solution was discarded, the wash was repeated as in step 3. 8. A volume of 100 µl of TMB one-step substrate reagent (Item H) was added to each well. And it was incubate for 30 minutes at room temperature in the dark with gentle shaking.

9. A volume of 50 µl of stop solution (Item I) was added to each well. Absorbance was read at 450 nm immediately.

Calculations

The standard curve of ACTIVIN A determination was plotted in Figure (2.9) and the ACTIVIN A level in each sample was determined. The OD is optical density.

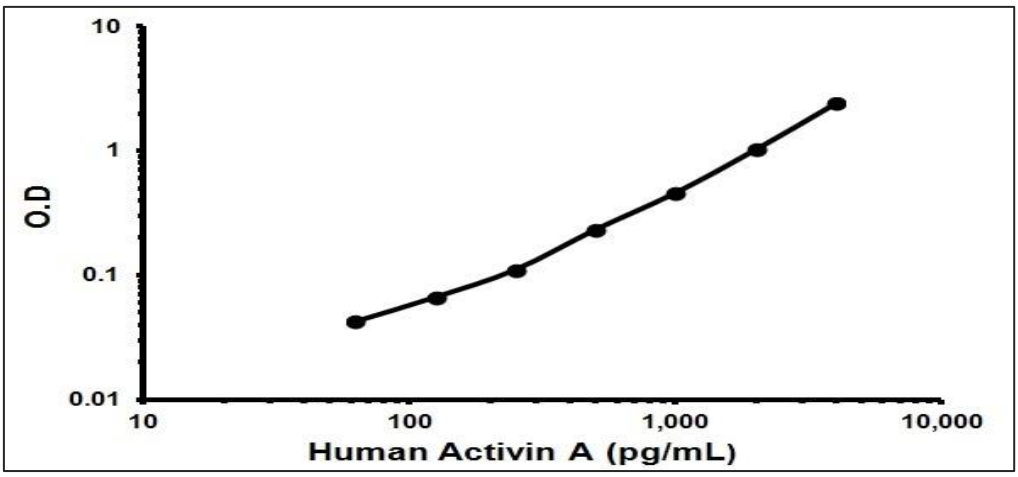

Figure 2.9 Standard curve of determination of ACTIVIN A concentration.

CHAPTER THREE

THE RESULTS

3. The Results

3.1 Biochemical parameters levels for patients women and control group

The study included 90 women in reproductive age, they were classified into two groups from (15-45) years old. In this research the relations of fertility hormones and recurrent miscarriages of 50 women suffering from recurrent miscarriages without risk factors diabetes mellitus, hypertension, heart disease, kidney disease, ectopic pregnancy, smoking and drugs and 40 women apparently healthy as a control group.

3.1.1 Comparison of groups' biochemical parameters

Independent T-test statistics were used for variables to compare between studies groups, and the results were expressed by mean± standard deviation (SD) and extraction P-value to show the difference variation . Comparing the serum of fertility hormones levels of INHIBIN B, ACTIVIN A, LH, FSH, prolactin, progesterone, ESTROGEN and testosterone between recurrent miscarriages and healthy subjects: the estimation of fertility hormones concentrations indicated INHIBIN B levels were significantly decreased (P=0.01) of patients, ACTIVIN A levels were significantly decreased (P=0.05)in patients when compared with control group, FSH level was insignificantly increased (P=0.02), LH level was insignificantly increased (P=0.01), prolactin level was significantly increased (P=0.05) of patients, progesterone level was insignificantly increased (P=0.05) in patients women when compared with control group, ESTROGEN level was significantly increased (P=0.01) of patients, testosterone level was insignificantly increased (P=0.01) of patients, as seen in the table(3.1).

Study the differences in the biomarkers level based on the age and BMI groups

The measurement of age level significantly increased of patients women, the BMI level almost equal (P=0.01) in patients women compared to the control group, as seen in the table (3.1).

Table 3.1: The concentration of ACTIVIN A, INHIBIN B, LH, FSH, ESTROGEN, progesterone, prolactin and testosterone of patients and control group.

parameters	subject	Mean± SD	P value
Age (Years)	Control	64.32 ± 12.38	0.05
	patients	67.16 ± 12.64	
BMI (kg/m^2)	Control	25.70 ± 4.85	0.01
	patients	25.94 ± 4.60	
INHIBIN B (pg/ml)	Control	133.64 ±10.00	0.01
	patients	115.56 ± 10.93	
ACTIVIN A (pg/ml)	Control	262.52 ± 8.54	0.05
	patients	240.57 ± 10.60	
FSH (mlU/ml)	Control	6.14 ± 1.64	0.02
	patients	7.01 ± 1.98	
LH (mlU/ml)	Control	5.71 ±1.85	0.01
	patients	7.52± 2.12	
Prolactin(mlU/ml)	Control	12.02 ± 6.58	0.05
	patients	15.02 ± 8.62	
Progesterone(ng/ml)	Control	0.53 ± 0.17	0.05
	patients	0.54 ± 0.18	
Estrogens (pg/ml)	Control	37.92 ± 13.45	0.01
	patients	51.82± 18.15	
Testosterone(ng/ml)	Control	0.60 ±0.20	0.01
	patients	1.25± 0.90	

BMI: Body Mass Index ; FSH: Follicle stimulating hormone ; LH: Luteinizing hormone ; SD: Standard deviation ; P-value: Probability level of statistical ; N.S: t-test p- value ≥ 0.05; No. of patients group=50; No. of control group=40

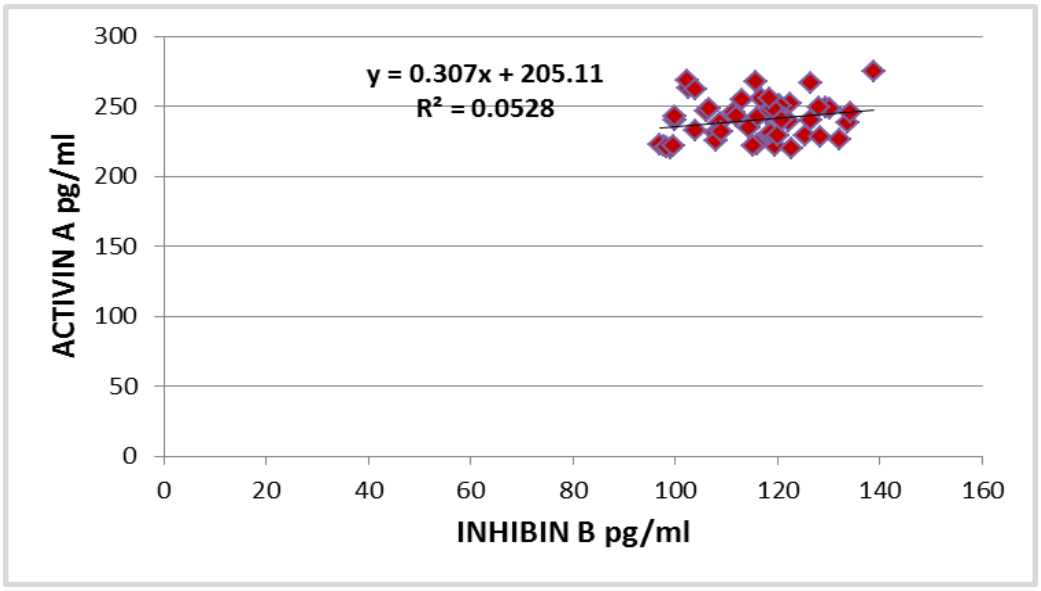

Figure (3.1) : Correlation coefficient between INHIBIN B concentration with ACTIVIN A concentration in patients women with recurrent miscarriages (r = 0.22) .

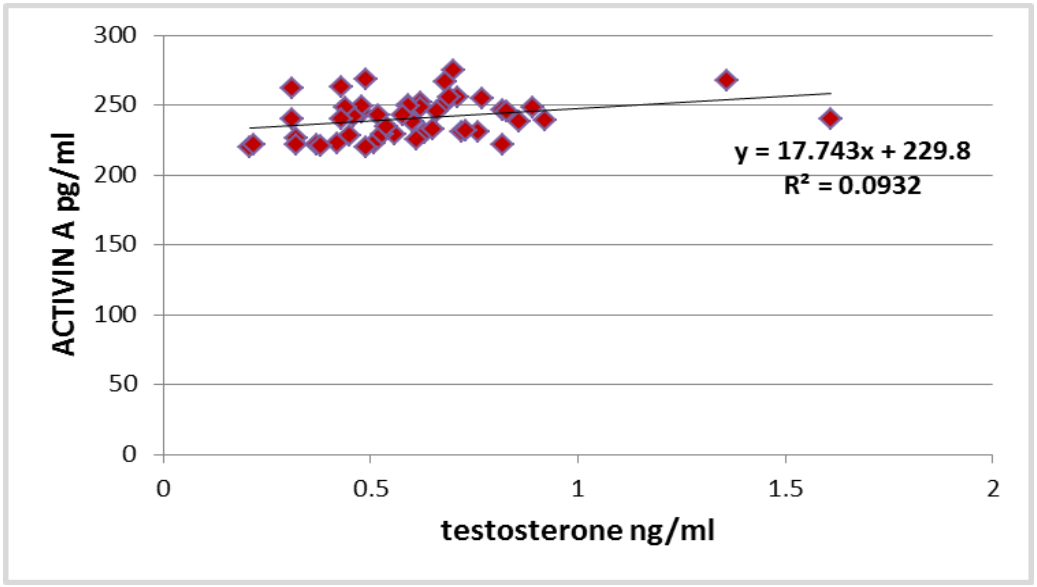

Figure (3.2) : Correlation coefficient between testosterone concentration with ACTIVIN A concentration in patients women with recurrent miscarriages (r = 0.3).

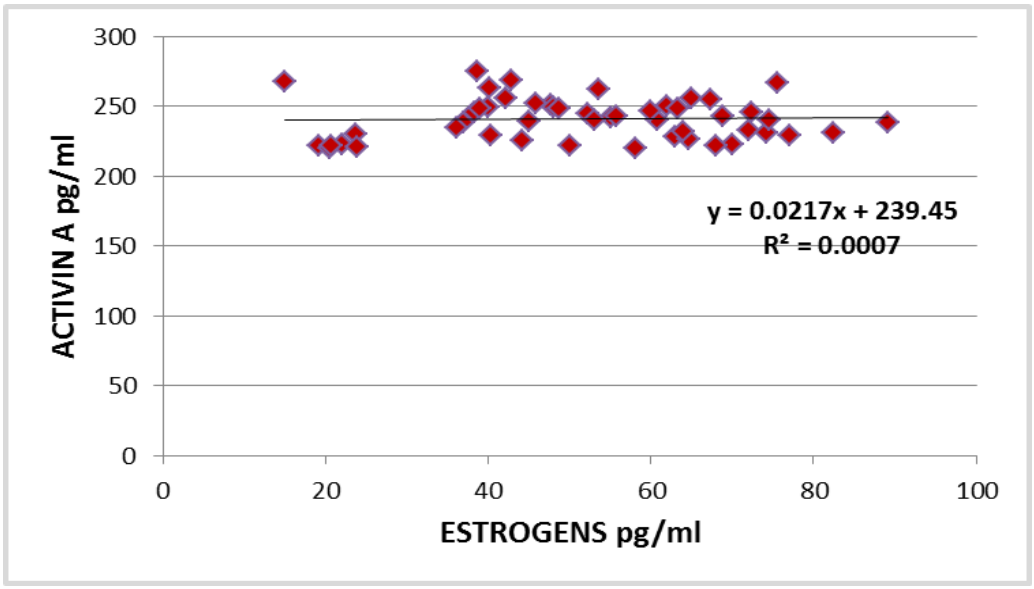

Figure (3.3) : Correlation coefficient between ESTROGEN concentration with ACTIVIN A concentration in patients women with recurrent miscarriages (r = 0.026).

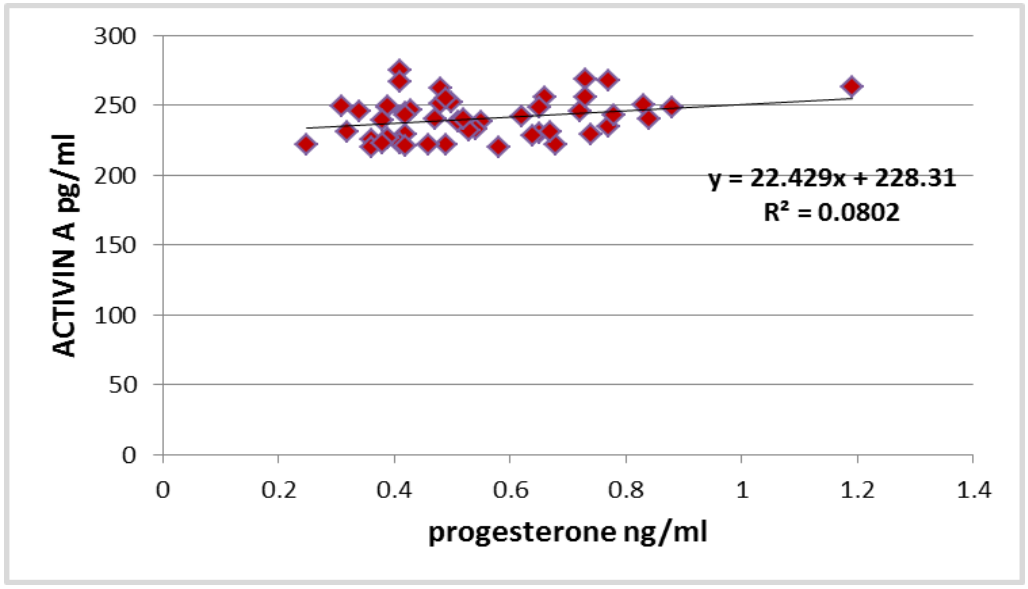

Figure (3.4) : Correlation coefficient between progesterone concentration with ACTIVIN A concentration in patients women with recurrent miscarriages (r = 0.28).

3.1.2 Comparison in fertility hormones levels between groups for age (15-29) years

Independent T-test statistics were used for variables to compare between studies groups, and the results were expressed by mean± standard deviation (SD) and the P-value to show the difference variation. Comparing the serum of fertility hormones levels of INHIBIN B, ACTIVIN A, LH, FSH, prolactin, progesterone, ESTROGEN and testosterone between recurrent miscarriages and healthy subjects for age (15-29) years. The estimation of fertility hormones concentration indicated INHIBIN B and ACTIVIN A levels were significantly decreased (P=0.01) of patients, FSH and LH insignificantly increased (P=0.01) of patients, prolactin level was insignificantly increased (P=0.04), progesterone level was insignificantly increased (P=0.02), ESTROGEN level was significantly increased (P=0.01), testosterone level was insignificantly increased (P=0.05) in patients compared to the control group, as seen in the table (3.2).

Study the differences in the biomarkers level based on the age and BMI groups

The measurement of age level was insignificantly increased , and the levels of body mass index (BMI) of the patients women showed a insignificantly decreased (P=0.01) compared to the control group, as seen in the table (3.2).

Table 3.2: The concentration of ACTIVIN A, INHIBIN B, LH, FSH, ESTROGEN, progesterone, prolactin and testosterone of patients and control group for age (15-29) years.

parameters	subject	Mean± SD	P Value
Age (Years)	Control (15-29)	60.95 ± 12.61	0.05
	Patients (15-29)	61.95 ± 10.76	
BMI (kg/m^2)	Control (15-29)	24.51 ± 4.80	0.01
	Patients (15-29)	23.65 ± 3.99	
INHIBIN B (pg/ml)	Control (15-29)	135.87 ±6.69	
	Patients (15-29)	117.00 ± 9.93	0.01
ACTIVIN A (pg/ml)	Control (15-29)	259.60 ± 8.42	
	Patients (15-29)	240.55 ± 15.46	0.01
FSH (mlU/ml)	Control (15-29)	5.24 ± 1.47	0.01
	Patients (15-29)	7.67 ± 1.89	
LH (mlU/ml)	Control (15-29)	5.58 ±2.20	
	Patients (15-29)	7.39± 2.21	0.01
Prolactin (mlU/ml)	Control (15-29)	12.17 ± 4.36	0.04
	Patients (15-29)	16.40 ± 8.86	
Progesterone(ng/ml)	Control (15-29)	0.45 ± 0.14	0.02
	Patients (15-29)	0.57 ± 0.19	
Estrogens (pg/ml)	Control (15-29)	37.77 ± 16.54	0.01
	Patients (15-29)	54.02 ± 16.80	
Testosterone (ng/ml)	Control(15- 29)	0.57 ± 0.20	0.05
	Patients (15-29)	0.63 ± 0.57	

BMI: Body Mass Index ; FSH: Follicle stimulating hormone ; LH: Luteinizing hormone ; SD: Standard deviation ; P-value: Probability level of statistical ; N.S: t-test p- value ≥ 0.05; No. of patients group=50; No. of control group=40

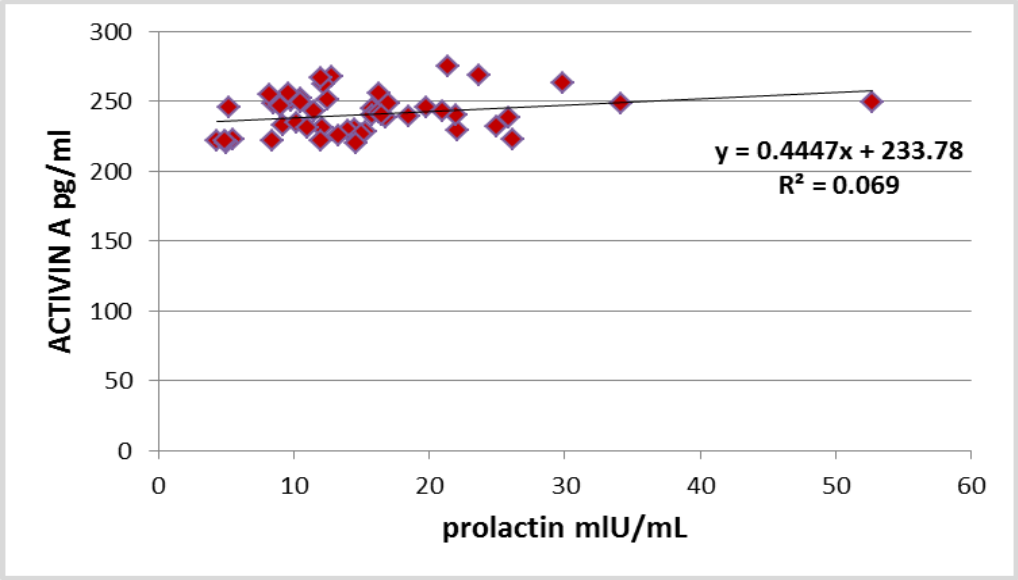

Figure (3.5) : Correlation coefficient between prolactin concentration with ACTIVIN A concentration in patients women with recurrent miscarriages (r = 0.26).

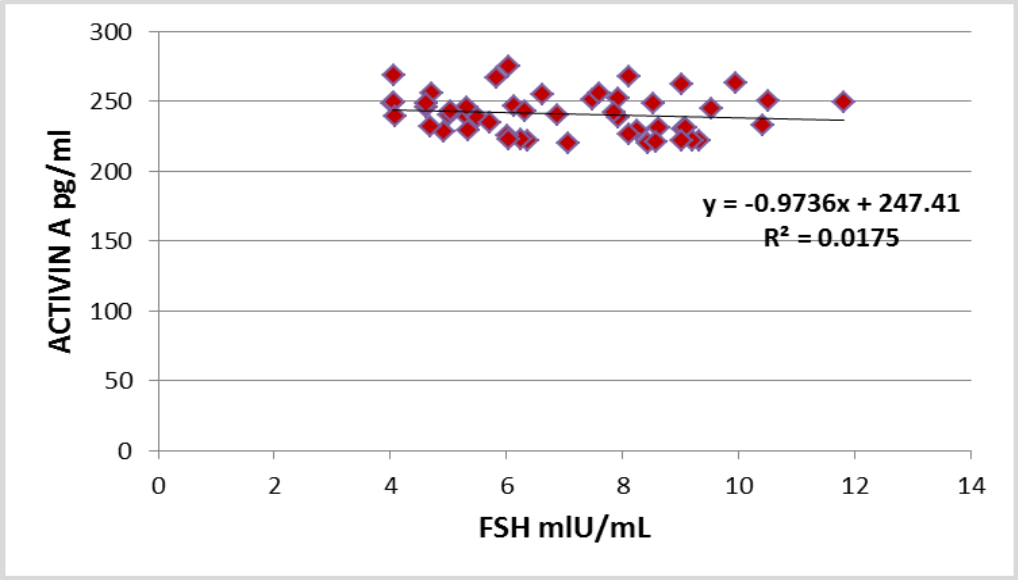

Figure (3.6) : Correlation coefficient between FSH concentration with ACTIVIN A concentration in patients women with recurrent miscarriages (r = 0.13).

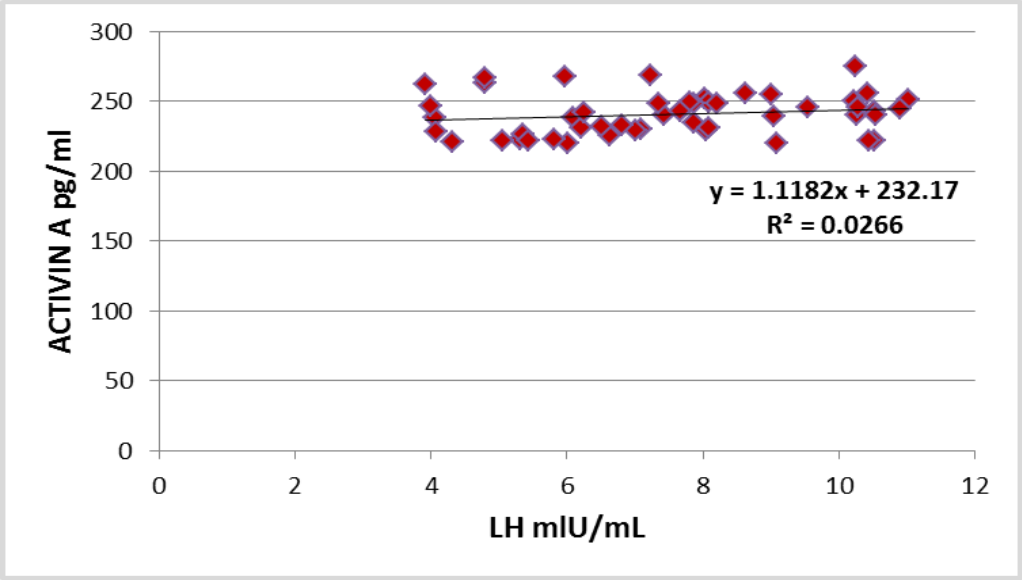

Figure (3.7) : Correlation coefficient between LH concentration with ACTIVIN A concentration in patients women with recurrent miscarriages (r = 0.16).

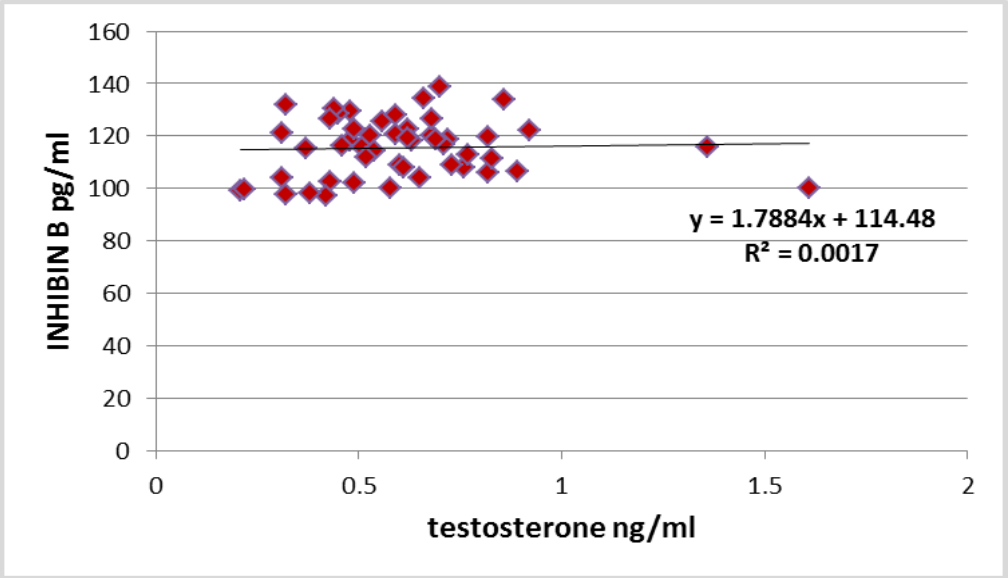

Figure (3.8) : Correlation coefficient between testosterone concentration with INHIBIN B concentration in patients women with recurrent miscarriages (r = 0.041).

3.1.3 Comparison in fertility hormones levels between groups for age (30-45) years

Independent T-test statistics were used for variables to compare between studies groups, and the results were expressed by mean± standard deviation (SD) and extraction P-value to show the difference variation . Comparing the serum of fertility hormones levels of INHIBIN B, ACTIVIN A, LH, FSH, prolactin, progesterone, ESTROGEN and testosterone between recurrent miscarriages and healthy subjects for age (30-45) years. The measurement of fertility hormones concentrations indicated INHIBIN B and ACTIVIN A levels were significantly decreased (P=0.01) of patients, FSH was insignificantly decreased (P=0.01), LH level was insignificantly increased (P=0.01), prolactin level was insignificantly increased (P=0.05) in patients when compared with control group, progesterone was significantly decreased (P=0.03), ESTROGEN level was significantly increased (P=0.04), testosterone level was significantly decreased (P=0.05) in patients compared to the control group, as seen in the table (3.3).

Study the differences in the biomarkers level based on the age and BMI groups

The measurement of age level significantly increased (P=0.03) in patients, and the levels of body mass index (BMI) of patients women showed a insignificantly increased (P=0.01) in patients compared to the control group, as seen in the table (3.3).

Table 3.3: The concentration of ACTIVIN A, INHIBIN B, LH, FSH, ESTROGEN, progesterone, prolactin and testosterone of patients and control group for age (30-45) years.

parameters	subject	Mean± SD	P Value
Age (Years)	Control (30-45)	68.44 ± 11.06	0.03
	Patients (30-45)	76.05 ±9.83	
BMI (kg/m^2)	Control (30-45)	27.15 ± 4.63	0.01
	Patients (30-45)	29.36 ±3.13	
INHIBIN B (pg/ml)	Control (30-45)	130.92 ±12.65	
	Patients (30-45)	113.39 ±12.22	0.01
ACTIVIN A (pg/ml)	Control (30-45)	266.09 ± 7.42	
	Patients (30-45)	240.62 ± 13.6	0.01
FSH (mlU/ml)	Control (30-45)	7.24 ± 1.08	0.01
	Patients (30-45)	6.03 ± 1.72	
LH (mlU/ml)	Control (30-45)	5.87 ±1.36	
	Patients (30-45)	7.70± 2.02	0.01
Prolactin (mlU/ml)	Control (30-45)	11.84 ± 8.71	0.05
	Patients (30-45)	13.61 ± 8.18	
Progesterone(ng/ml)	Control (30-45)	0.63 ± 0.17	0.03
	Patients (30-45)	0.51 ± 0.16	
Estrogens (pg/ml)	Control (30-45)	38.10 ± 8.77	0.04
	Patients (30-45)	48.52 ± 19.99	
Testosterone (ng/ml)	Control (30-45)	0.62 ± 0.20	0.05
	Patients (30-45)	0.60 ± 0.56	

BMI: Body Mass Index ; FSH: Follicle stimulating hormone ; LH: Luteinizing hormone ; SD: Standard deviation ; P-value: Probability level of statistical ; N.S: t-test p- value ≥ 0.05; No. of patients group=50; No. of control group=40

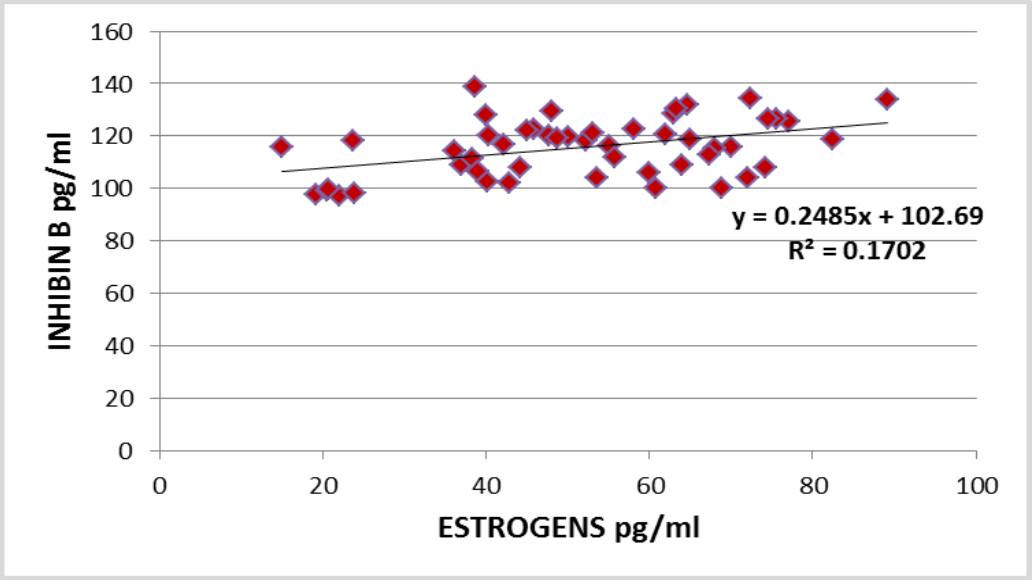

Figure (3.9) : Correlation coefficient between ESTROGEN concentration with INHIBIN B concentration in patients women with recurrent miscarriages (r = 0.41).

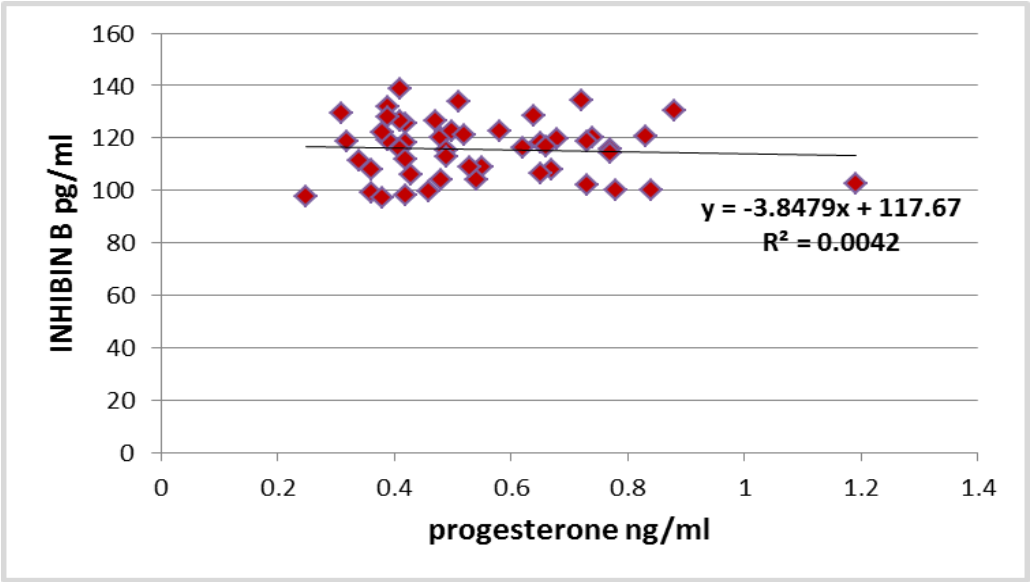

Figure (3.10) : Correlation coefficient between progesterone concentration with INHIBIN B concentration in patients women with recurrent miscarriages (r = 0.064).

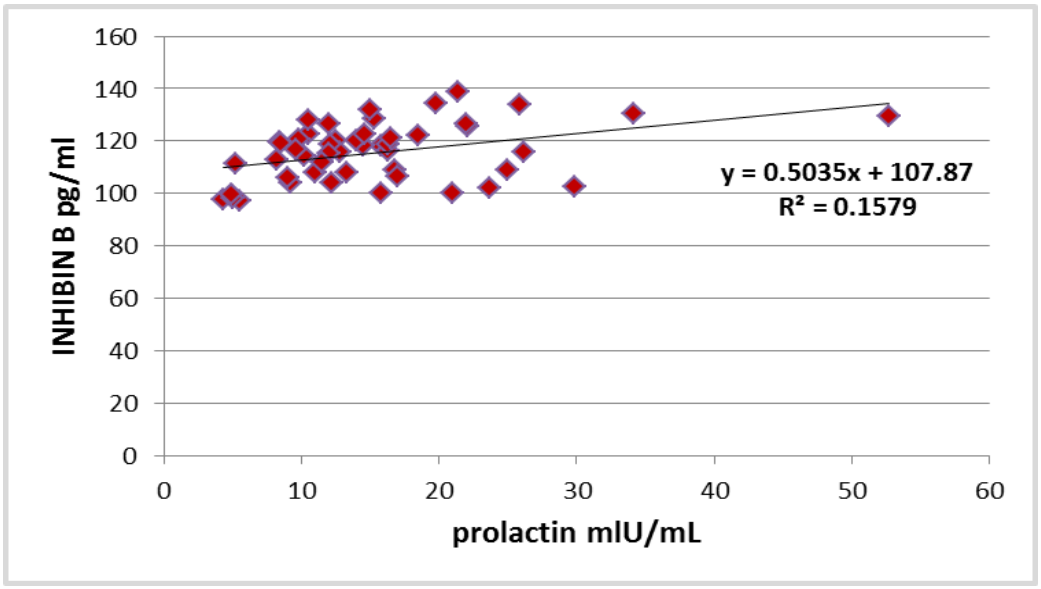

Figure (3.11) : Correlation coefficient between prolactin concentration with INHIBIN B concentration in patients women with recurrent miscarriages (r = 0.39) .

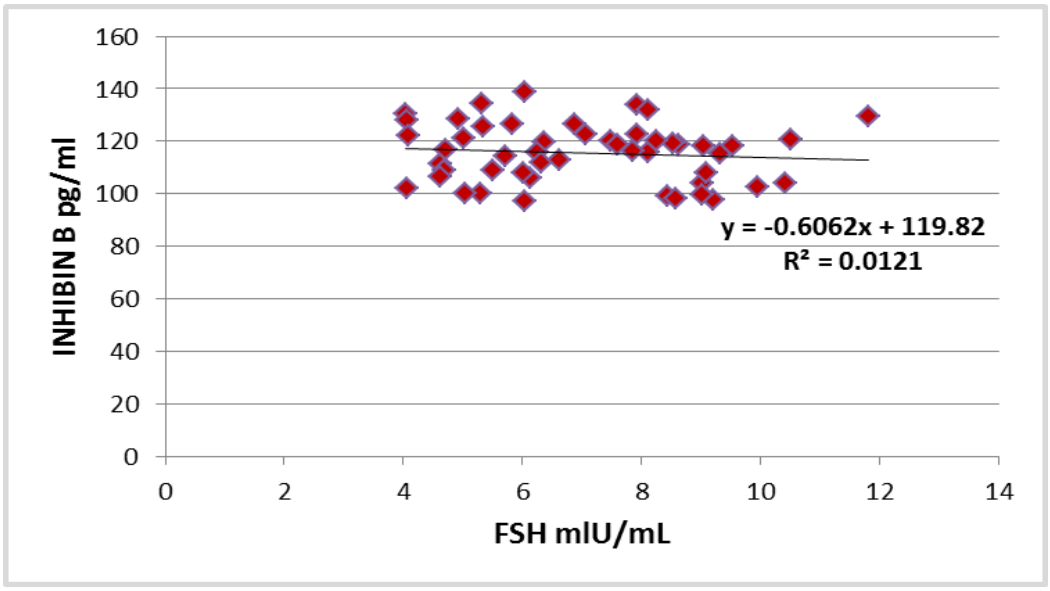

Figure (3.12) : Correlation coefficient between FSH concentration with INHIBIN B concentration in patients women with recurrent miscarriages (r = 0.11).

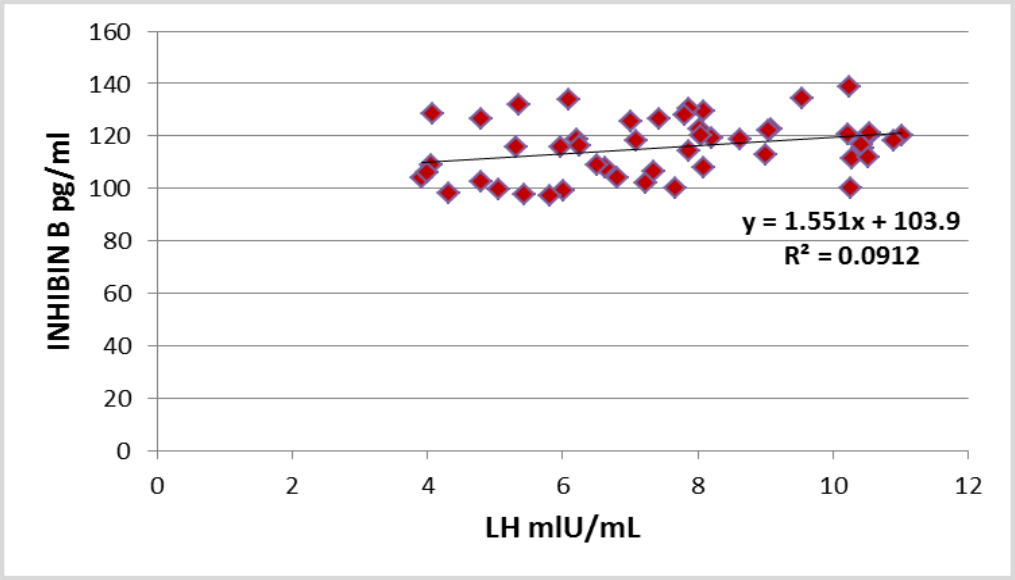

Figure (3.13) : Correlation coefficient between LH concentration with INHIBIN B concentration in patients women with recurrent miscarriages (r = 0.301).

3.2 The effect of Body Mass Index (BMI) and biochemical parameters levels of patients women

Pie charts were used to describe the proportion of patients for each body mass index as normal weight 18.5-24.9 kg/m² has lowest percentage obtain 27% of patient groups , over weight (pre obesity) 25-29.9 kg/m² was 34% of patients and obesity over 30 kg/m² obtain higher percentage 39% of patients groups that's mean most of patient PCOS have high BMI, as shown in figure (3.14).

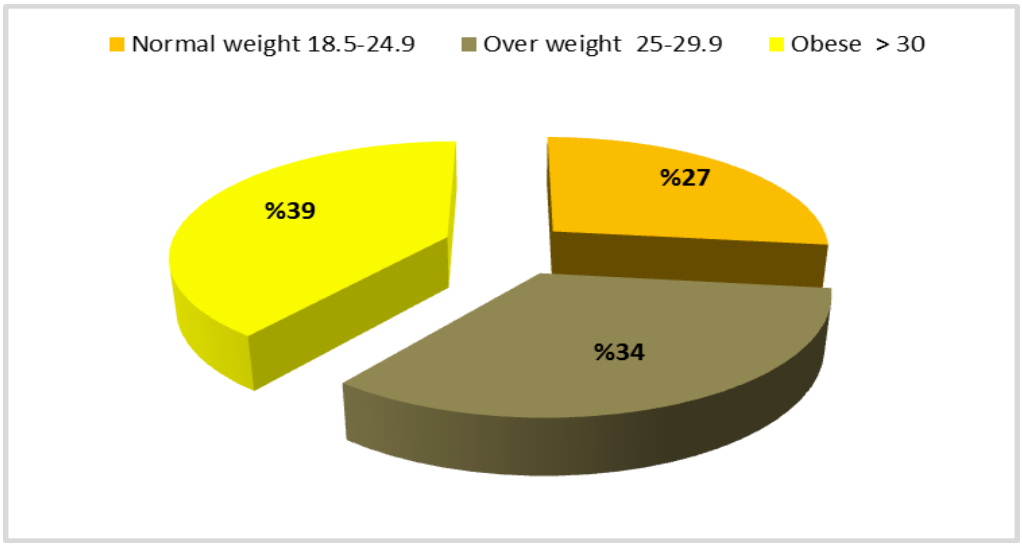

Figure (3.14) : BMI proportion in patients groups.

3.2.1 The effects of the BMI on the levels of ACTIVIN A and INHIBIN B concentration in patients and control group

The study found that the levels of ACTIVIN A and INHIBIN B concentration in control group were the highest in Body Mass Index (BMI), whether the women were of normal weight, over weight and obese when compared with patients women, as shown in figure (3.15) , (3.16).

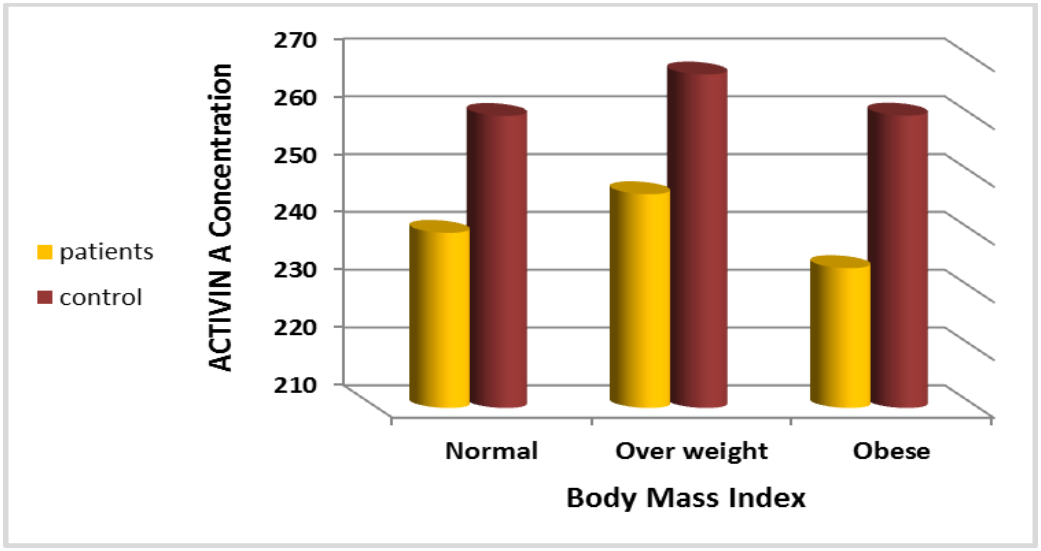

Figure (3.15) : Levels of BMI with ACTIVIN A concentration in patients and control group.

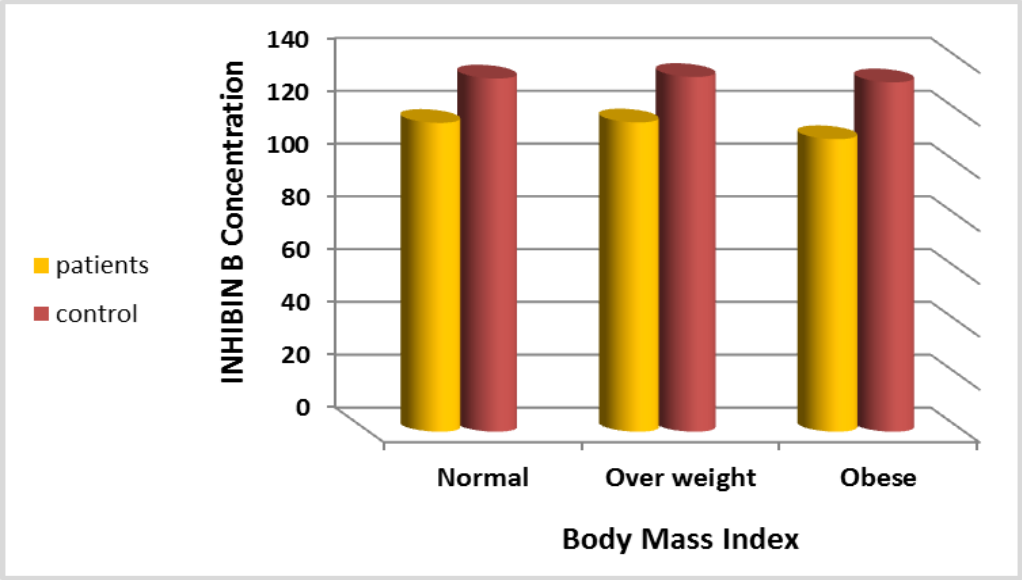

Figure (3.16) : Levels of BMI with INHIBIN B concentration in patients and control group.

3.2.2 The effects of the BMI on the levels of testosterone and progesterone concentration in patients and control group

The study found that the levels of testosterone and progesterone concentration in patients were the highest in Body Mass Index (BMI) of normal weight from control group when compared with the women of pre obesity(over weight) and obese, the level of BMI of control group in over weight and obese higher than patients women, as shown in figure (3.17),(3.18).

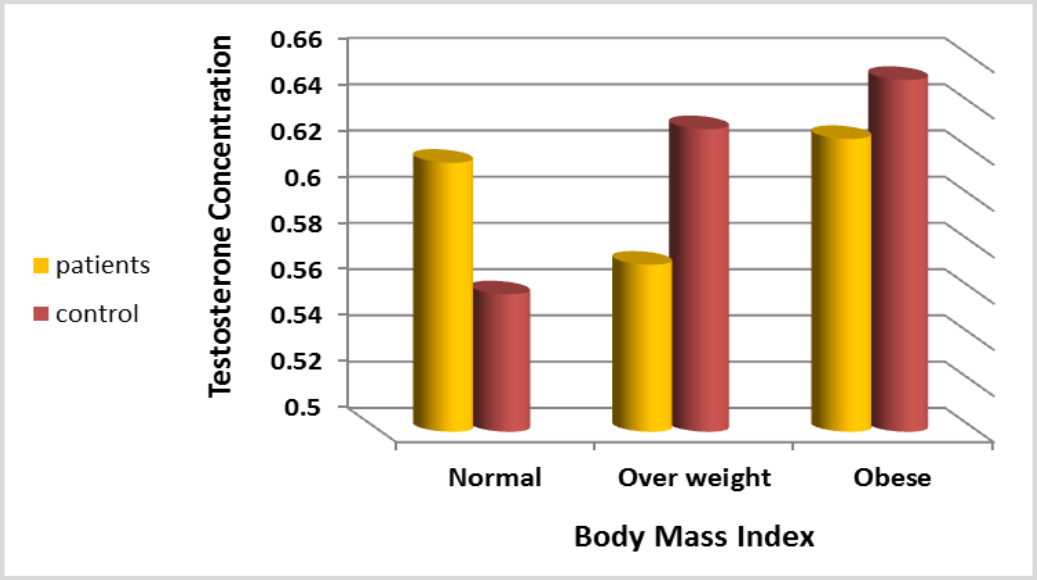

Figure (3.17) : Levels of BMI with testosterone concentration in patients and control group.

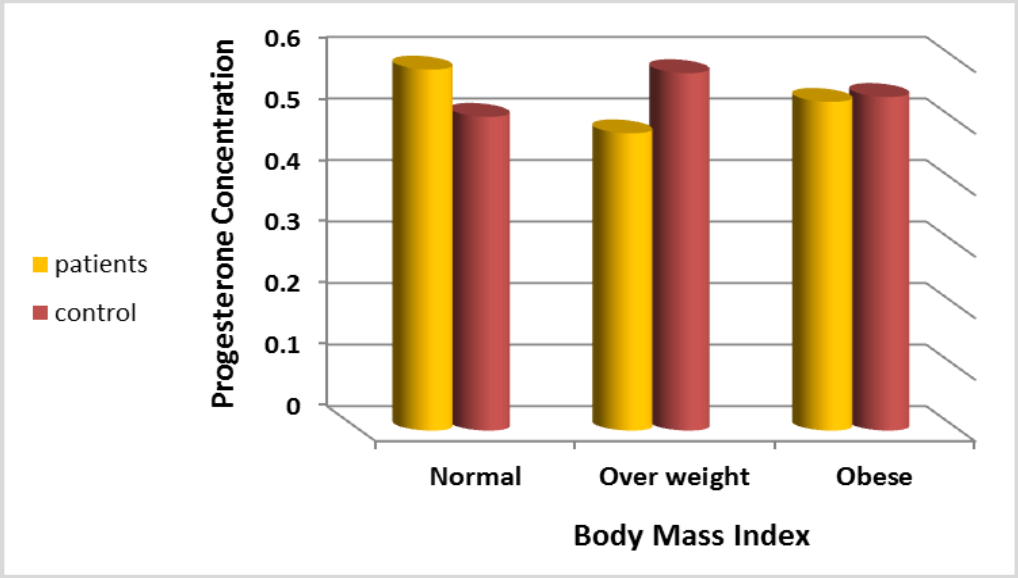

Figure (3.18) : Levels of BMI with progesterone concentration in patients and control group.

3.2.3 The effects of the BMI on the levels of ESTROGEN and prolactin concentration in patients and control group

The study found that the levels of ESTROGEN and prolactin concentration in patients were the highest in BMI normal and overweight

when compared with the obese women, the control group in obese was the higher BMI from than patients when compared with the BMI of normal and overweight women, as shown in figure (3.19),(3.20).

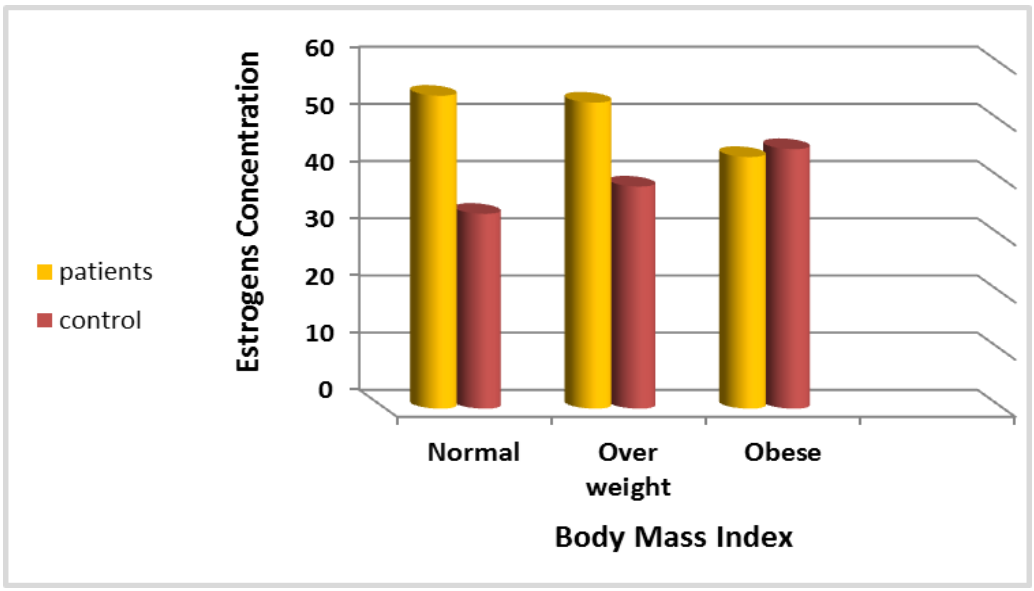

Figure (3.19) : Levels of BMI with ESTROGEN concentration in patients and control group.

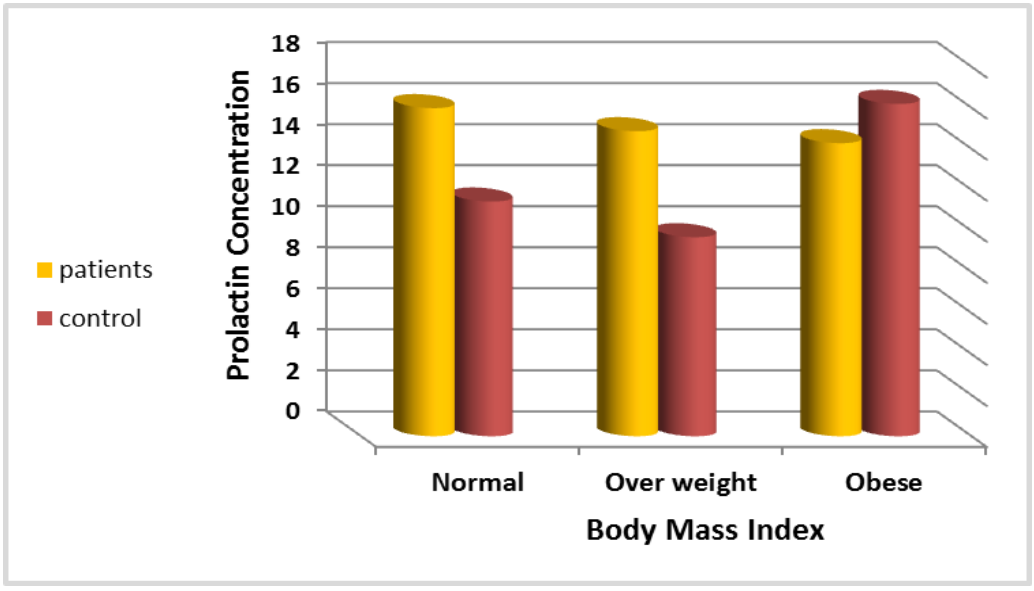

Figure (3.20) : Levels of BMI with prolactin concentration in patients and control group.

3.2.4 The effects of the BMI on the levels of FSH concentration in patients and control group

The measurement of the level of FSH concentration in patients shown the higher BMI in normal and obese when compared with overweight, while in control group shown higher BMI of FSH level in overweight than normal and obese weight, as shown in figure (3.21).

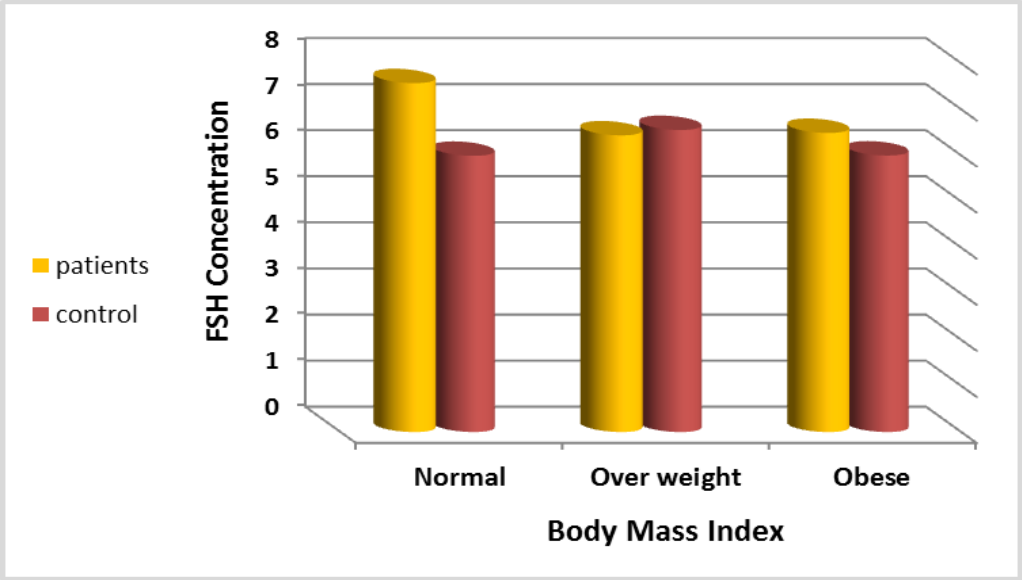

Figure (3.21) : Levels of BMI with FSH concentration in patients and control group.

3.2.5 The effects of the BMI on the levels of LH concentration in patients and control group

The measurement of the level of LH concentration in patients women shown the highest BMI level in normal, overweight and obese compared to the control group, as seen in the figure (3.22).

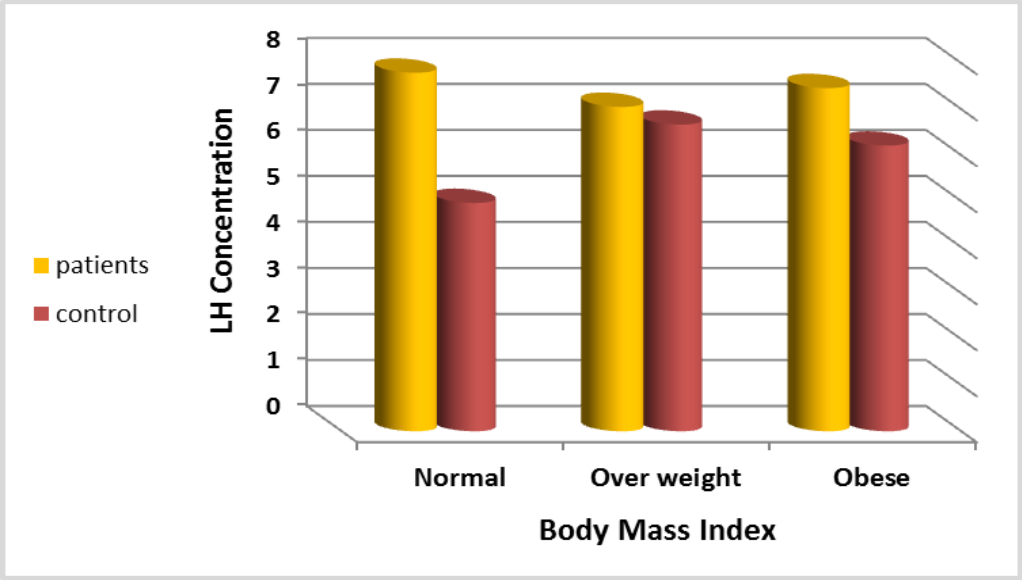

Figure (3.22) : Levels of BMI with LH concentration in patients and control group.

3.3 The effect of the duration of miscarriage on biochemical parameters levels in patients group

The relation between duration of miscarriage and concentration of biochemical parameters of patients, and its effect on level of fertility hormones in one month, two months and three months or more, for the purpose of comparing between hormones.

3.3.1 The levels of ACTIVIN A hormone within duration of miscarriage in patients women

The results of the research showed that the level of ACTIVIN A concentration for patients women with the duration of miscarriage, where the one month was more miscarriage than the three and two months, as shown in figure (3.23).

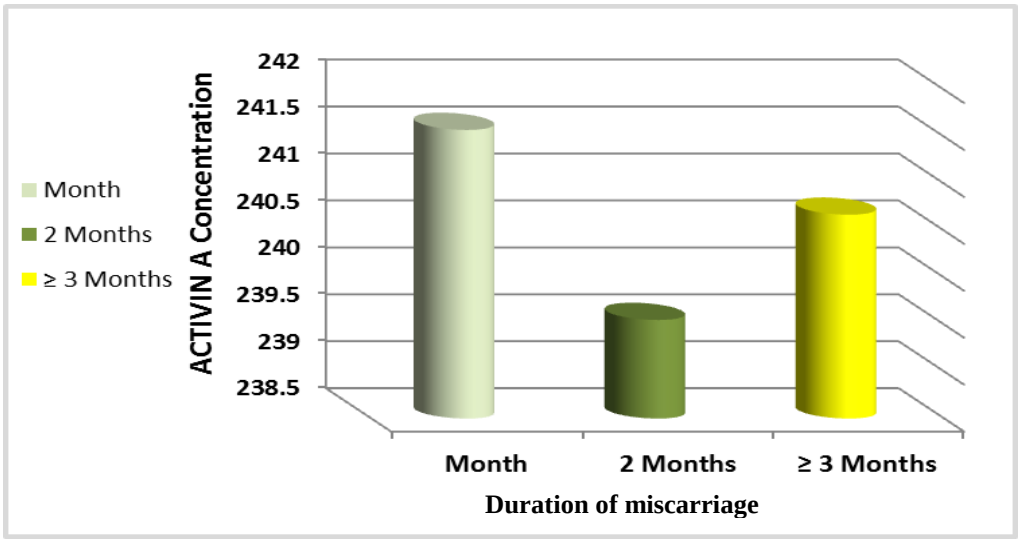

Figure (3.23) : The relation of duration of miscarriage with ACTIVIN A concentration.

3.3.2 The levels of INHIBIN B, ESTROGEN and LH hormones within duration of miscarriage in patients women

In this study , the measurement of the levels of INHIBIN B, ESTROGEN and LH concentration in patients with duration of miscarriage, showed that the levels of these hormones were higher in one month when compared with two and three months, as shown in figures (3.24-26) .

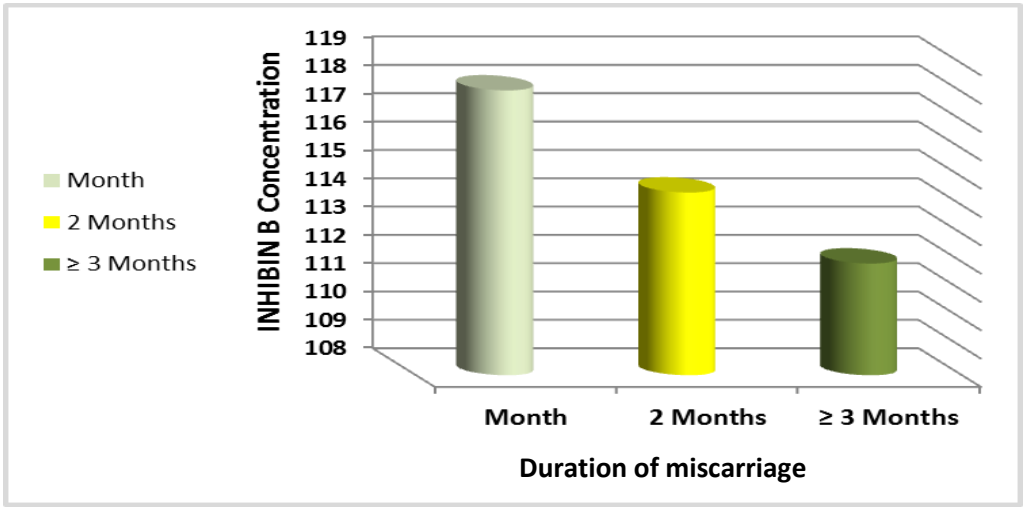

Figure (3.24) : The relation of duration of miscarriage with INHIBIN B concentration.

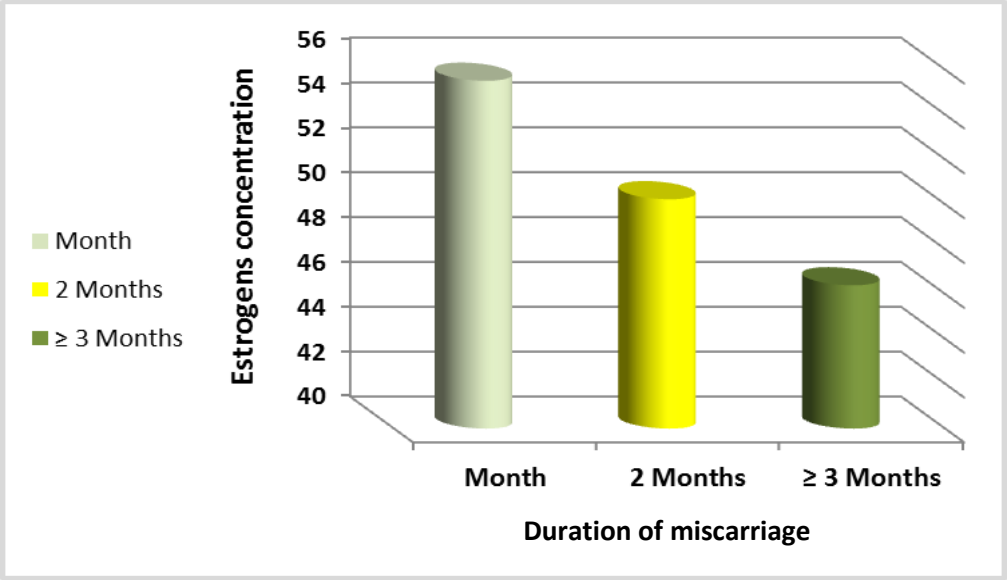

Figure (3.25) : The relation of duration of miscarriage with ESTROGEN concentration.

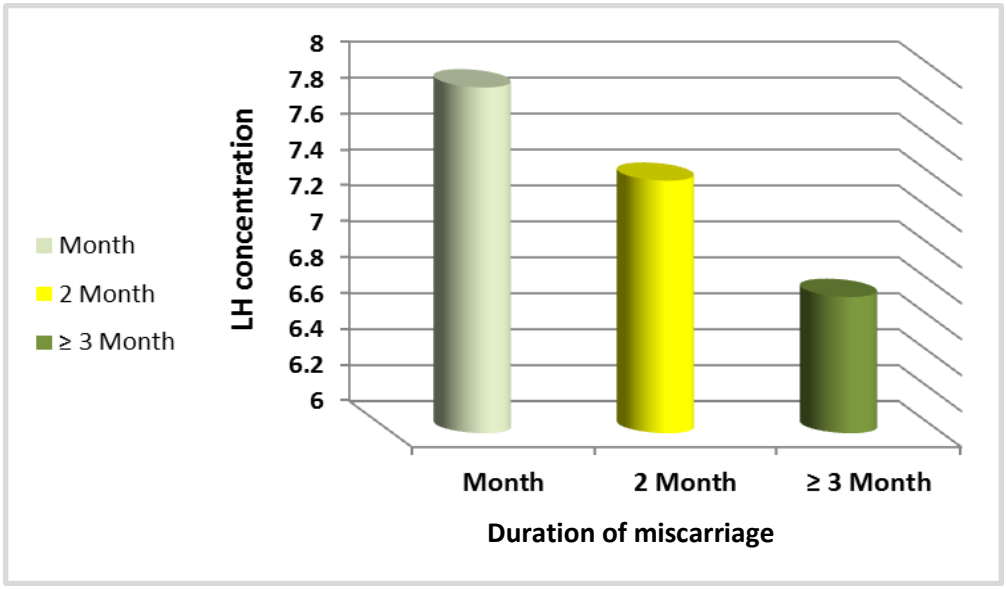

Figure (3.26) : The relation of duration of miscarriage with LH concentration.

3.3.3 The levels of prolactin, progesterone, FSH and testosterone hormones within duration of miscarriage in patients women

The measurement of this hormones with duration of miscarriage notice the more miscarriages in three months or more from pregnancy

than one and two months from embryo age, as shown in figures (3.27-30).

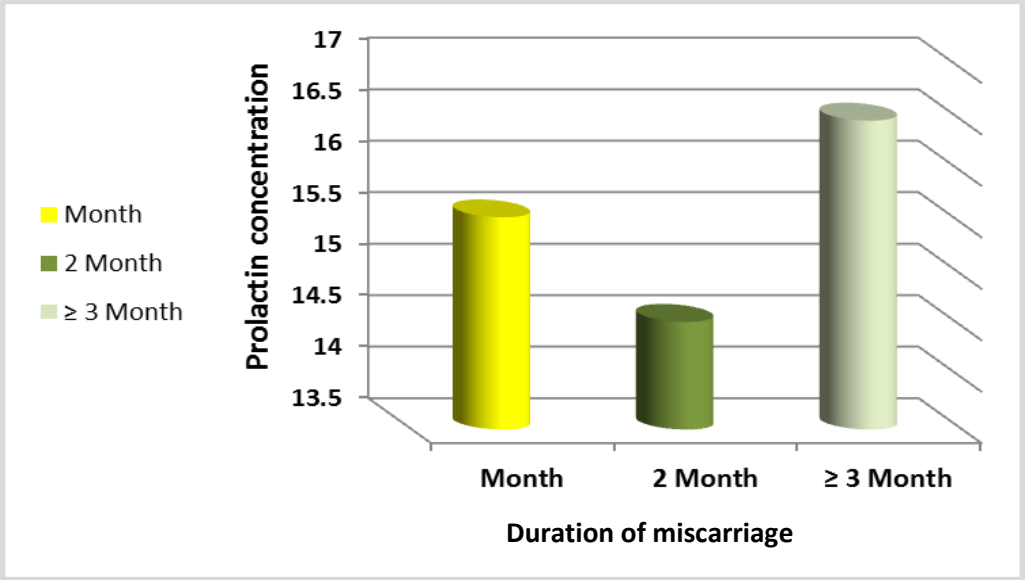

Figure (3.27) : The relation of duration of miscarriage with prolactin concentration.

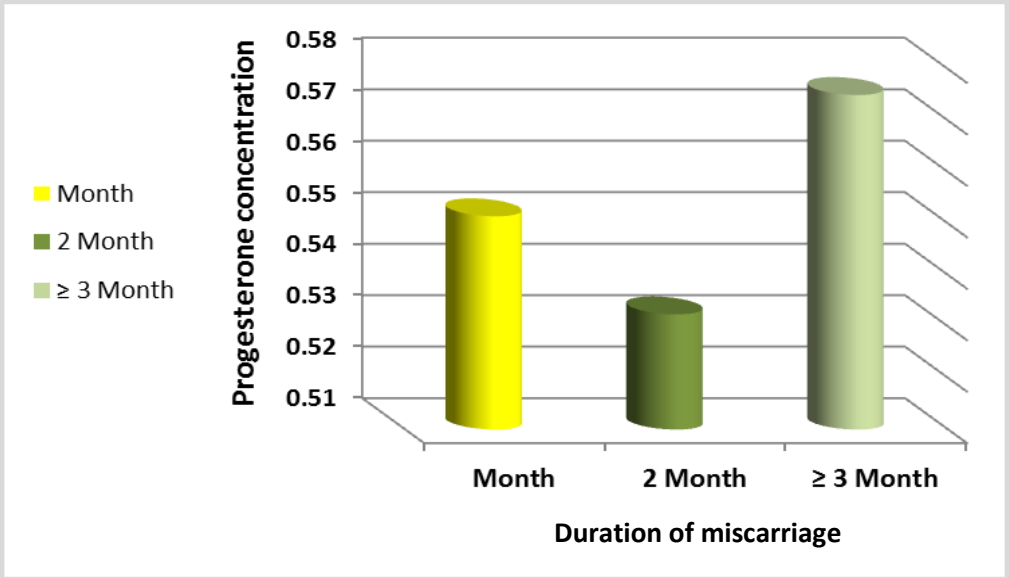

Figure (3.28) : The relation of duration of miscarriage with progesterone concentration.

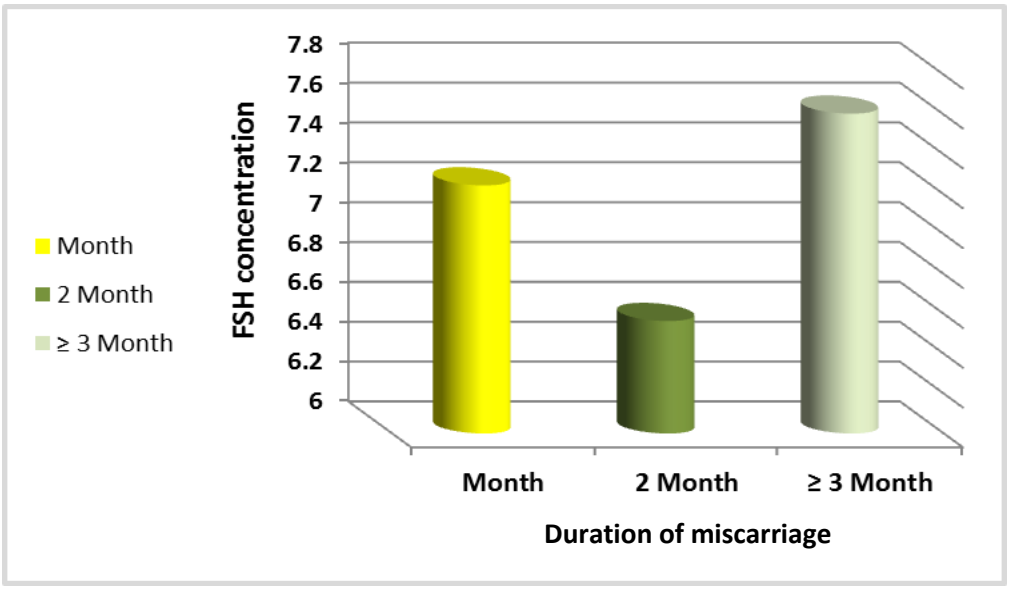

Figure (3.29) : The relation of duration of miscarriage with FSH concentration.

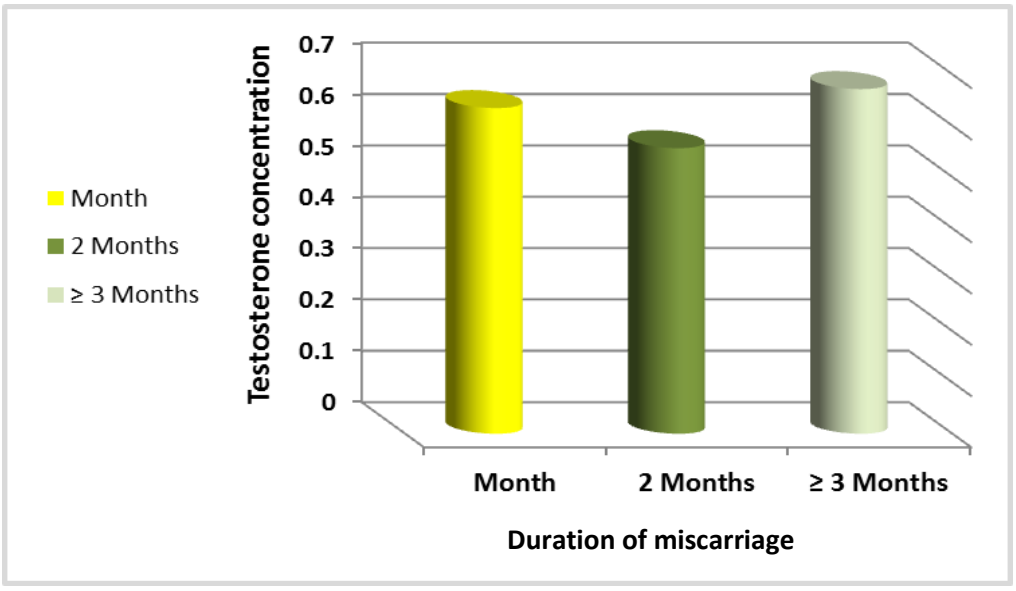

Figure (3.30) : The relation of duration of miscarriage with testosterone concentration.

3.4 The effect of number of miscarriages on fertility hormones concentration in patients women

Patients group was categorized according to the number of miscarriages into women with two, three or more miscarriages and its

relation with the level of fertility hormones to find out the difference between the hormones.

3.4.1 The effect of the number of miscarriages on the level of ACTIVIN A, ESTROGEN, FSH, progesterone and testosterone concentration in patients

In this study, it was found that the concentration of these hormones with the number of miscarriages for patients women , that women with two recurrent miscarriages have a higher percentage than other women with the number of miscarriages of three or more, as shown in figures (3.31-35).

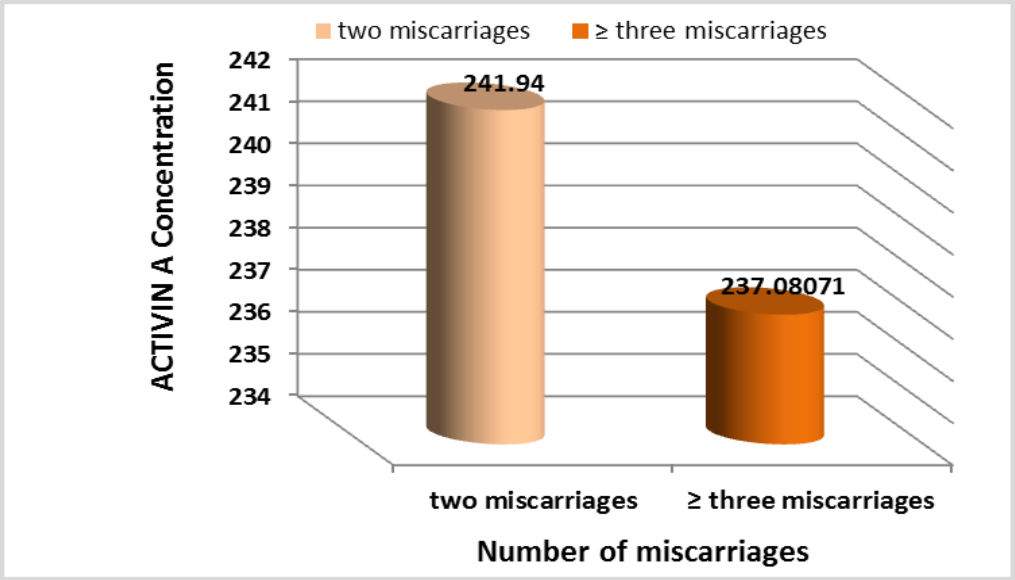

Figure (3.31) : The relation of number of miscarriages with ACTIVIN A concentration.

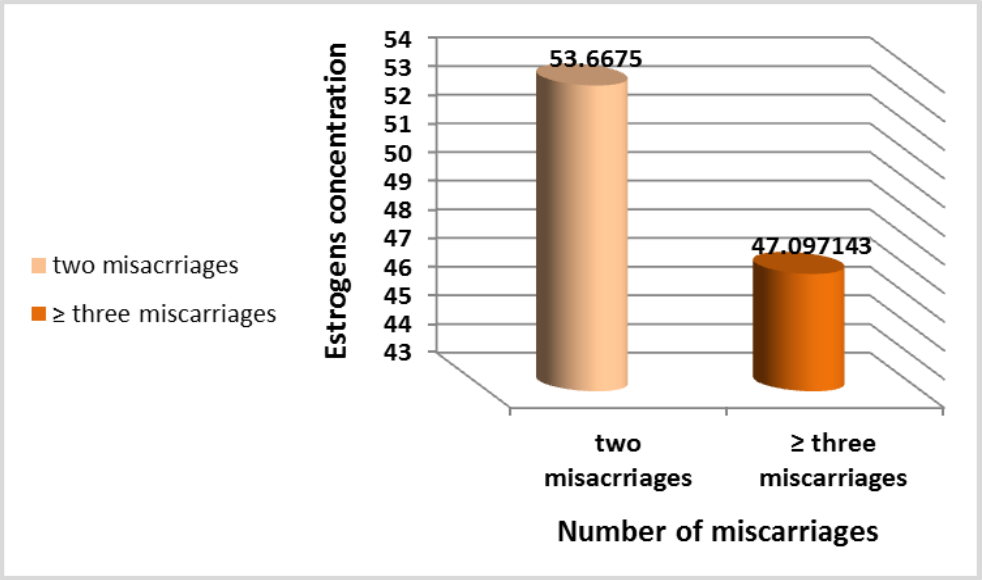

Figure (3.32) : The relation of number of miscarriages with ESTROGEN concentration.

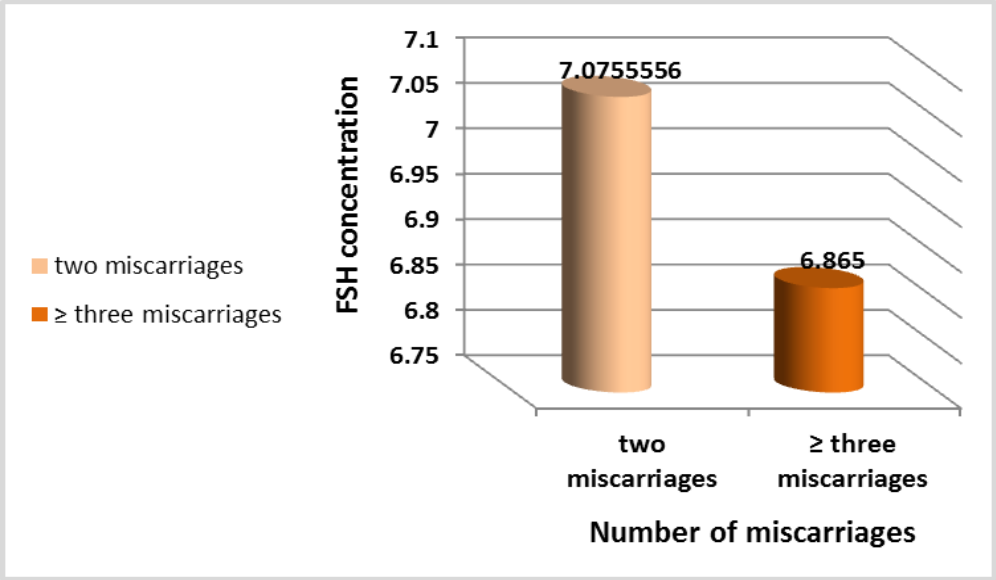

Figure (3.33) : The relation of number of miscarriages with FSH concentration.

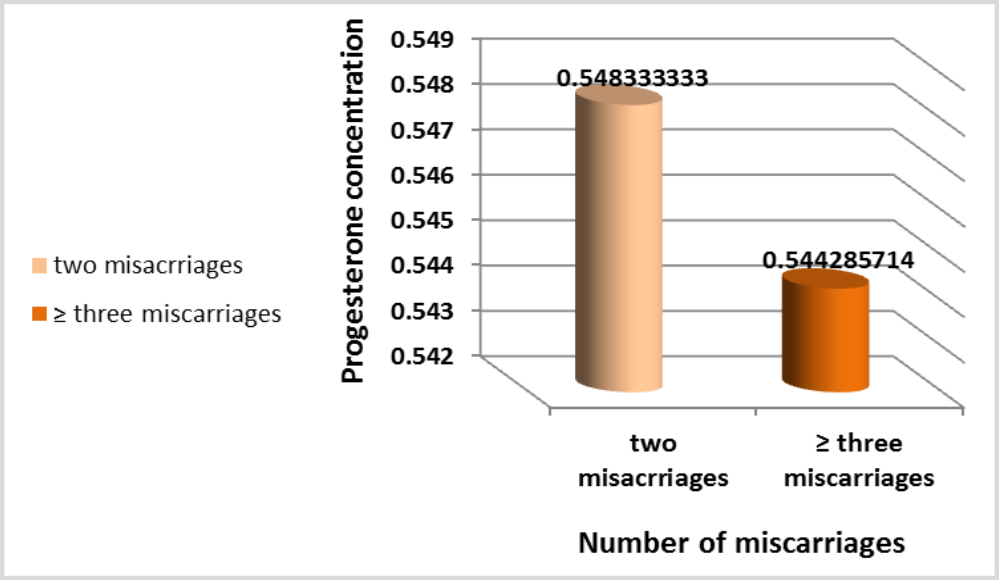

Figure (3.34) : The relation of number of miscarriages with progesterone concentration.

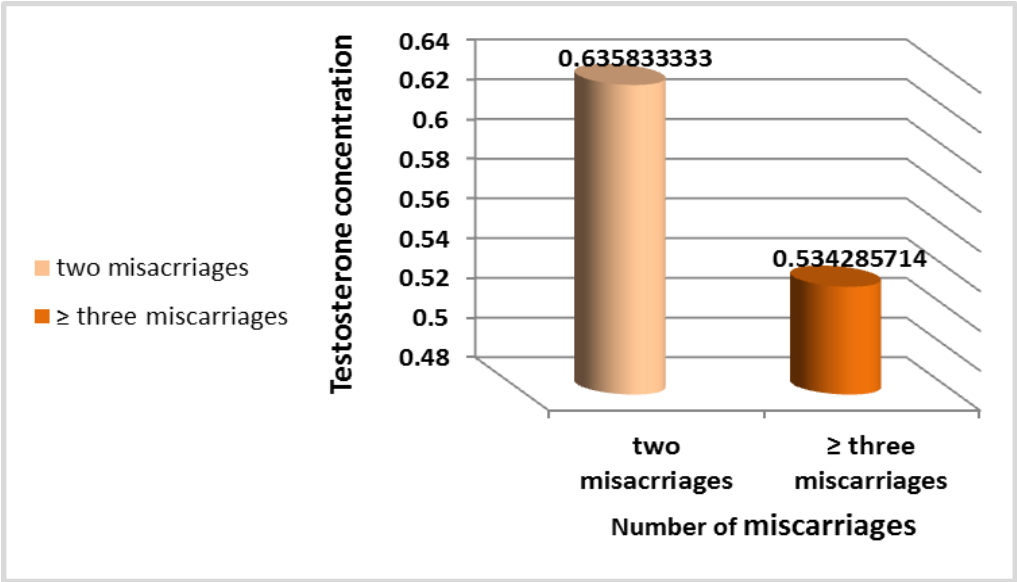

Figure (3.35) : The relation of number of miscarriages with testosterone concentration.

3.4.2 The effect of the number of miscarriages on the level of INHIBIN B, LH and prolactin concentration in patients

The measurement of the concentration of these hormones with the number of miscarriages, it was found that the level of hormones in

women with three miscarriages or more is higher than in women with two recurrent miscarriages, as shown in figures (3.36-38).

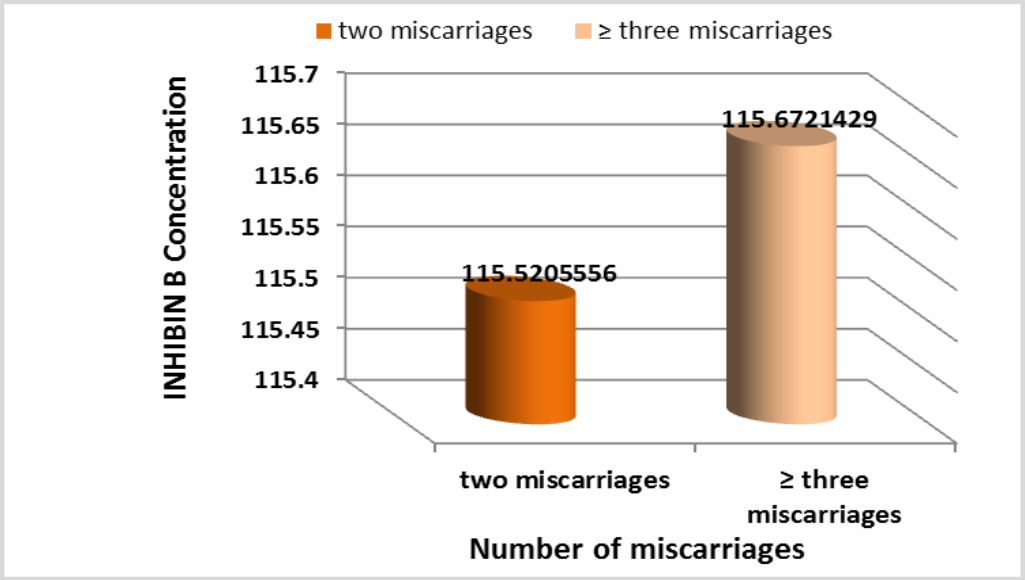

Figure (3.36) : The relation of number of miscarriages with INHIBIN B concentration.

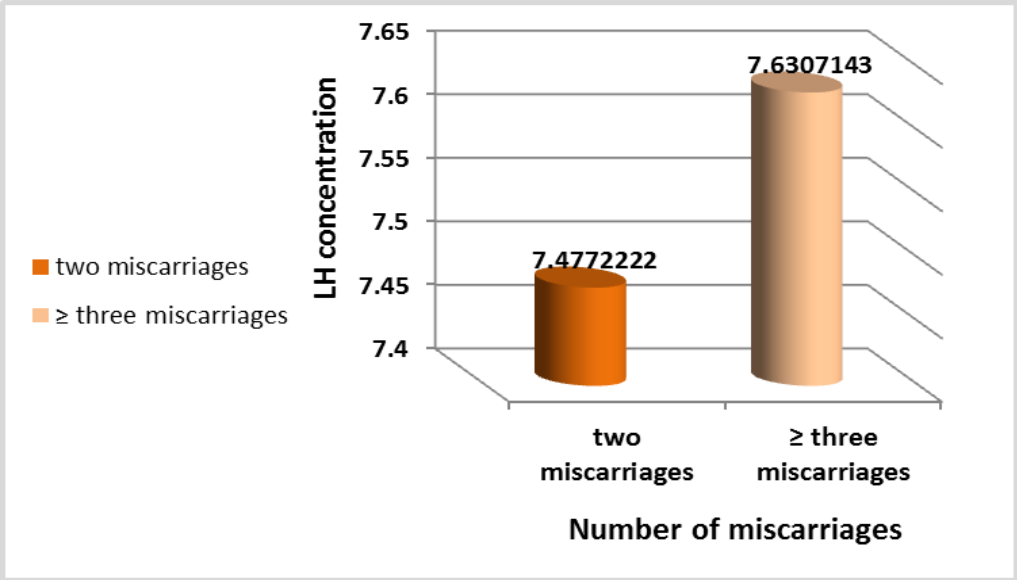

Figure (3.37) : The relation of number of miscarriages with LH concentration.

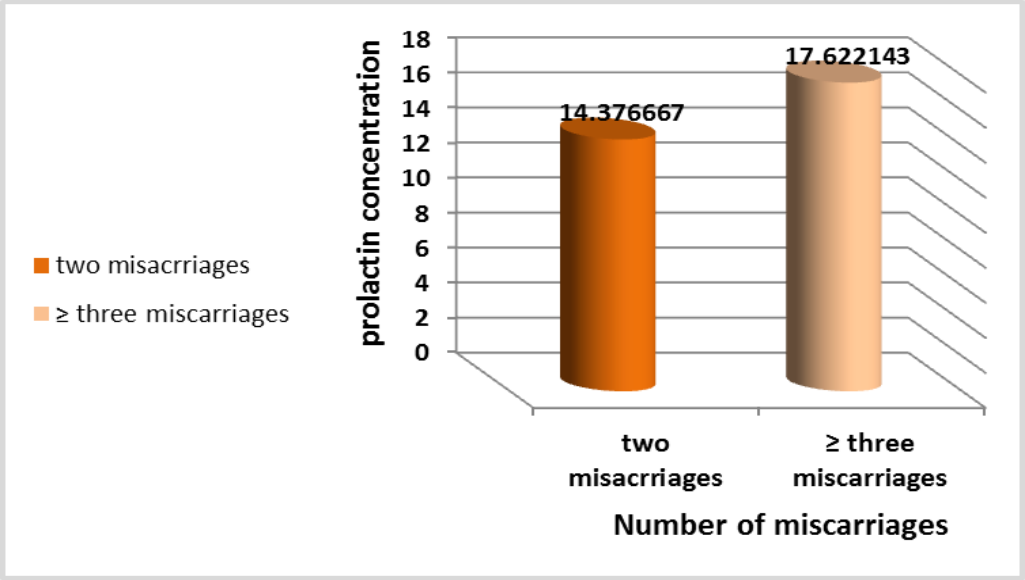

Figure (3.38) : The relation of number of miscarriages with prolactin concentration.

3.5 The effect of last miscarriages date on biochemical parameter concentration

The group of women patients was divided according to the last miscarriages date into three groups, the first group the last miscarriage (1-4)months, the second group from (5-9)months, and the third group more than (9months -2years) and the relation with the concentration of fertility hormones for the purpose of knowing their effect on the level of these hormones.

3.5.1 The effect of last miscarriages date on the level of ACTIVIN A, FSH, progesterone and testosterone concentration

The study found that the level of concentration of these hormones was higher percentage in first group(1-4 months) for the last miscarriages date for patients women when compared with the second group (5-9 months)and third group (over 9 months - 2 years), as shown in figures (3.39-42).

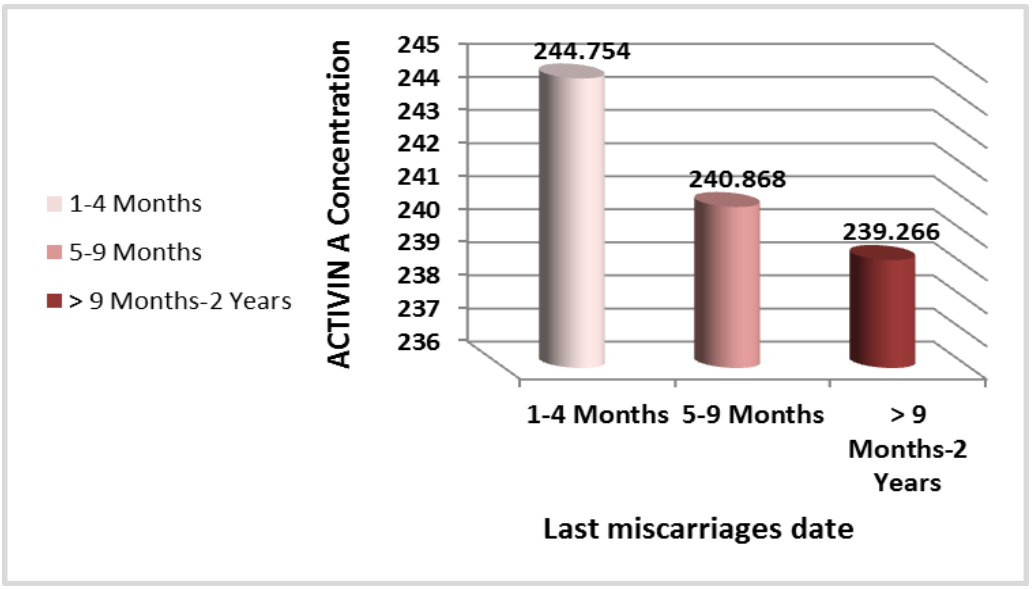

Figure (3.39) : The relation of last miscarriages date with ACTIVIN A concentration.

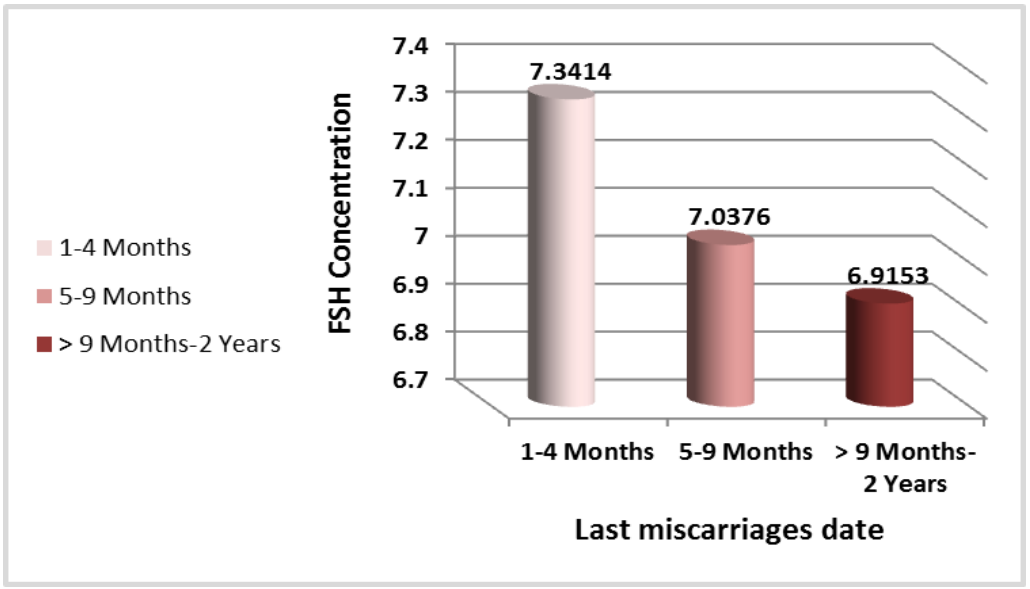

Figure (3.40) : The relation of last miscarriages date with FSH concentration.

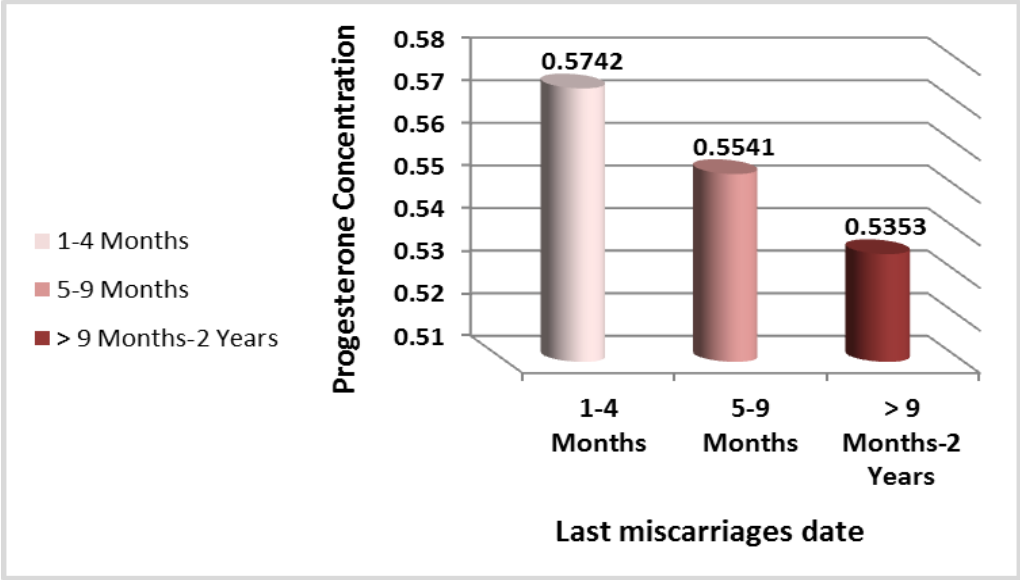

Figure (3.41) : The relation of last miscarriages date with progesterone concentration.

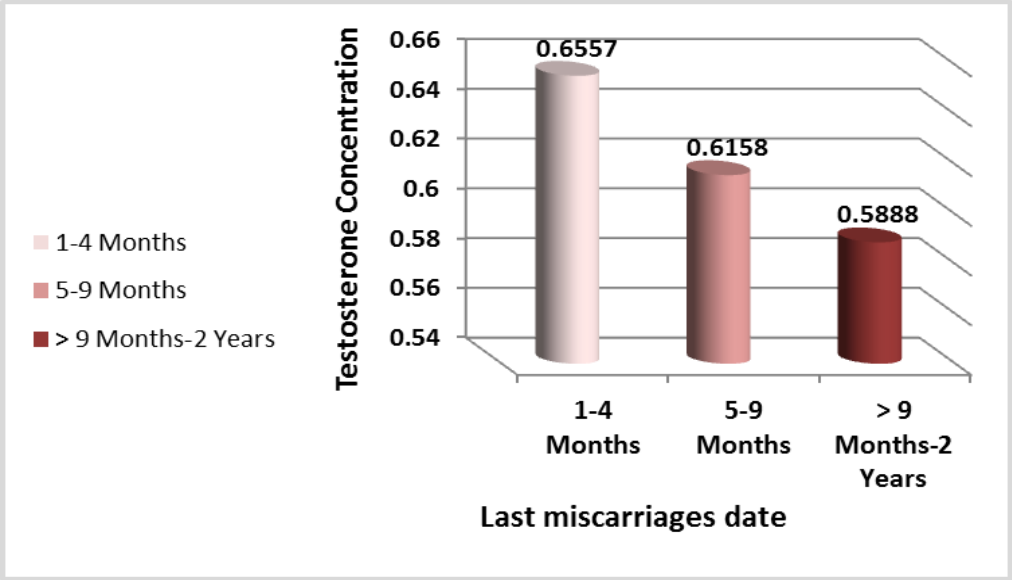

Figure (3.42) : The relation of last miscarriages date with testosterone concentration.

3.5.2 The effect of last miscarriages date on the level of INHIBIN B concentration

The measurement of the level of the INHIBIN B concentration with the last miscarriages date of patients ,showed insignificantly

decreased in the second group (5-9 months) when compared with the first group (1-4 months), as shown in figure (3.43).

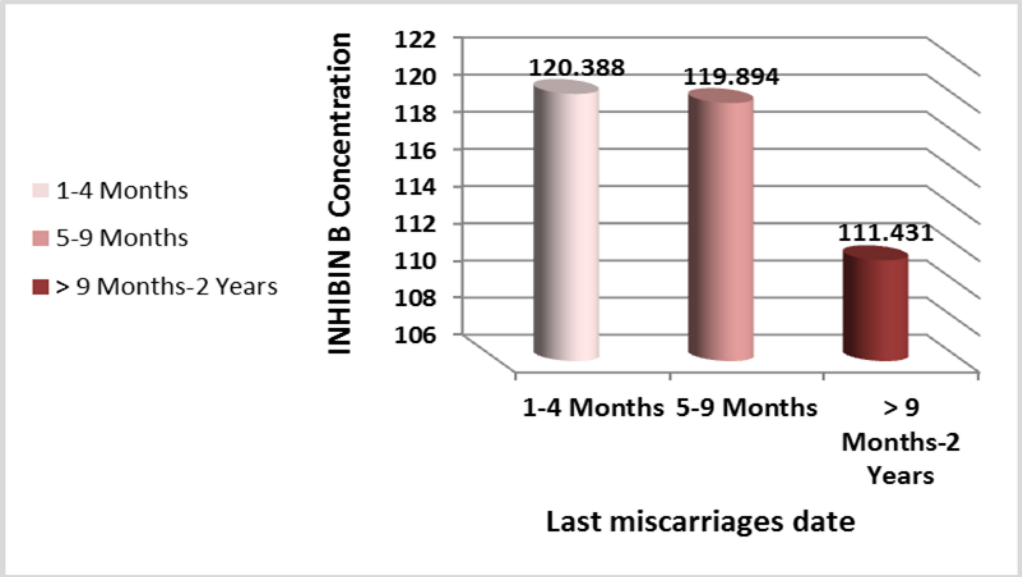

Figure (3.43) : The relation of last miscarriages date with INHIBIN B concentration.

3.5.3 The effect of last miscarriages date on the level of the ESTROGEN, LH and prolactin concentration

The study found that the level of concentration of these hormones was higher percentage in second group(5-9months) for the last miscarriages date for patients women when compared with the first group (1-4 months)and third group (over 9 months - 2 years), as shown in figures (3.44-46).

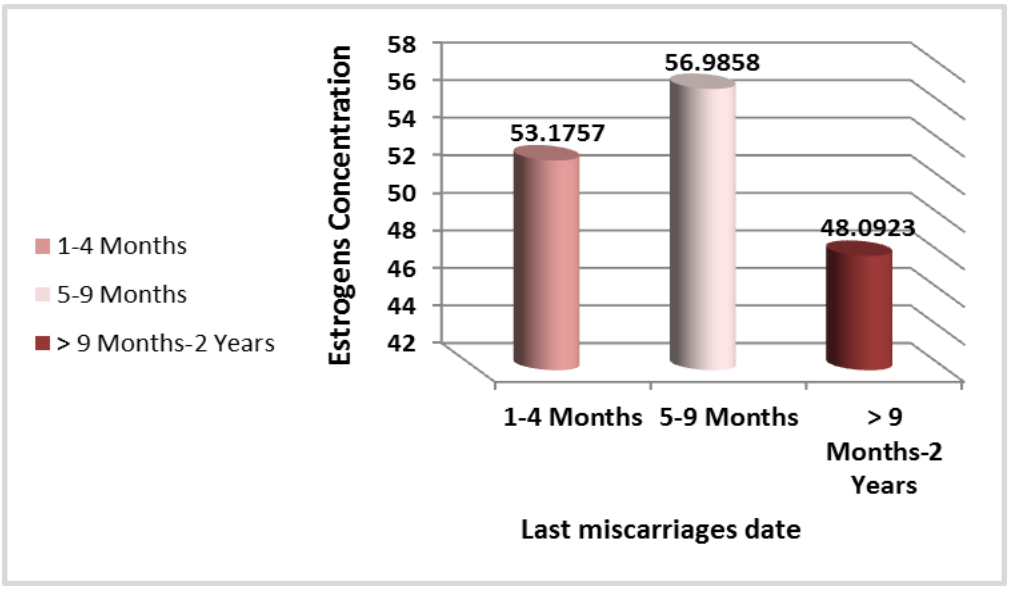

Figure (3.44) : The relation of last miscarriages date with ESTROGEN concentration.

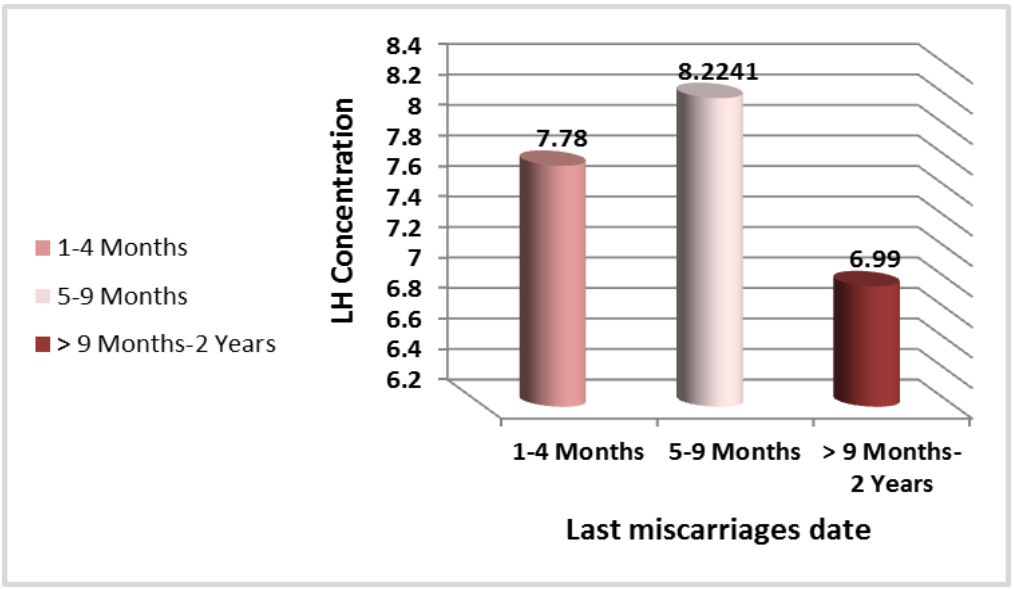

Figure (3.45) : The relation of last miscarriages date with LH concentration.

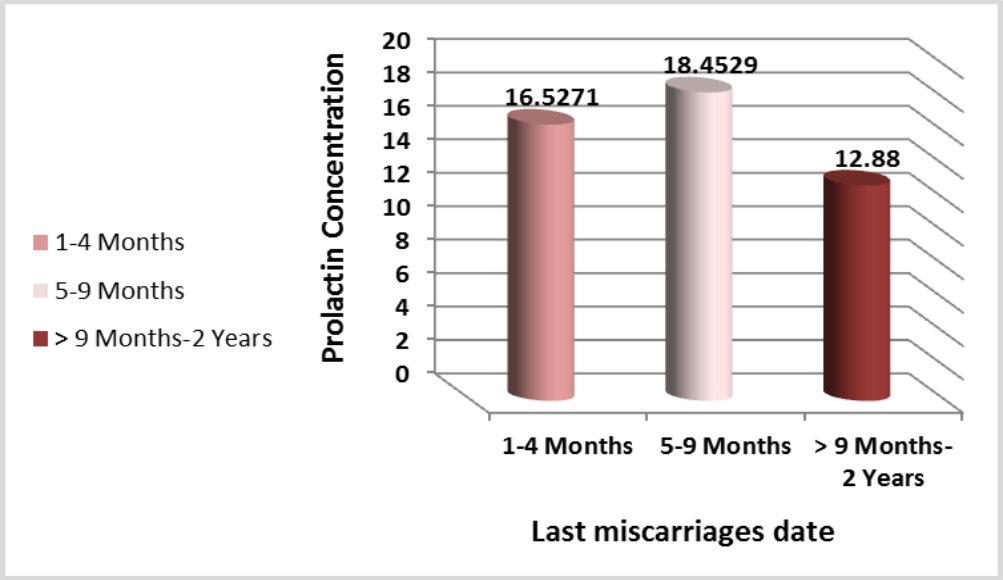

Figure (3.46): The relation of last miscarriages date with prolactin concentration.

CHAPTER FOUR

DISCUSSION

4.1 Physiological changes mechanisms in fertility hormones between age groups (15-45) years

The results of hormones in women with PCOS and control group indicated revealed that INHIBIN B and ACTIVIN A levels were decreased, but FSH, LH, ESTROGEN, prolactin, progesterone and testosterone levels were increased, as shown in table (3.1). Low levels of INHIBIN B have been linked to poor ovulation, low pregnancy rates, and an increased risk of miscarriage. Study suggests The amount of INHIBIN B relates with follicular function and oocyte quantity, suggesting that INHIBIN B cell product plays a role in follicular growth [147]. Another study found that The amount of both INHIBIN B proteins released into follicular fluid appears to rise with follicle growth, while concentrations may somewhat fall in the biggest follicles due to strength in a larger fluid volume [148]. INHIBIN B is a key indication of ovarian reserve (the ovary's ability to react to gonadotropin stimulation), predicting the size of retrievals and determining gonadotropin dose for Assisted Reproductive Technology [149]. Study found that INHIBIN B in follicular fluid was discovered to be a useful marker for follicular development. They also discovered a link between INHIBIN B levels in follicular fluid and embryo scores on the second or third day of their previous menstrual cycle, making INHIBIN B an effective predictor of embryo quality [150]. INHIBIN B levels decrease before blood FSH levels rise, making it a more sensitive indicator of ovarian age than FSH [151]. INHIBIN B levels were lower in patients using progesterone for polycystic ovarian syndrome [152]. Some studies showed that INHIBIN B concentration increases in patients with polycystic ovary syndrome [153]. Whereas some have demonstrated that it decreases [154].

ACTIVIN A levels in the blood are greater in pregnant women than in non-pregnant women, and they rise throughout the pregnancy until a round 28 weeks [155]. The use of serum ACTIVIN A as a predictor of pregnancy failure has sparked considerable controversy among researchers, with some claiming that ACTIVIN A readings can identify pregnant women at risk of missed miscarriages [156]. Measuring the ACTIVIN A hormone level important in treating early pregnancy difficulties in the first trimester of pregnancy in women who have bleeding during pregnancy that puts the foetus at risk of miscarriage [157]. **Asashima, et al in (1990)** found that although ACTIVIN A is present in the blood throughout pregnancy, the specific biological role of these proteins in pregnancy is, as yet, unknown [158]. A decrease in ACTIVIN A concentration or functional activity may induce PCOS-like symptoms [159]. The results are not concordant with the study by **Eldar-Geva, T., et al in (2001)** found that The serum ACTIVIN A ratio was shown to be higher in PCOS patients than in controls [160]. In women, the source of serum ACTIVIN A is uncertain, in response to FSH stimulation, peptide is produced by ovarian cells [161].

In the pathogenesis of polycystic ovarian disease, abnormality of the hypothalamic-pituitary-ovarian or adrenal axis has been suggested disturbance in the excretion type of the gonadotropin-releasing hormone results in the relative increase in LH - FSH release [162]. Ovulation does not occur in polycystic ovarian disease patients due to a high LH/FSH ratio [163]. In PCOS women, higher serum LH and the LH:FSH ratio are common [164]. **Cook et al in (2002)** Found that the women with PCOS showed greater serum LH levels than women without the condition [165]. One study found that the variability in the LH/FSH ratio is at least as large in healthy women as it is in those with clinical PCOS [166,167]. In

30% of PCOS patients, a little increase in blood prolactin levels can be found in both the follicular and luteal phases [168].

According to research, an increase in blood prolactin levels might lead to a decrease in ovarian follicles and ovulation [169]. Early studies showed that the patients with polycystic ovaries had higher prolactin serum levels in early investigations. Recent studies that used repeated serum samples to rule out transitory prolactin spikes found a less common link between these diseases [170,171]. A study found that high blood prolactin levels were not connected with PCOS and could not be regarded a characteristic of the condition; nonetheless, the need to evaluate serum prolactin levels in PCOS patients has been issued [172]. Other studies have also agreed that monitoring blood prolactin levels in PCOS patients is a fine decision [173,174].

Infertility and spontaneous abortion are most commonly caused by a lack of luteal phase progesterone production and activity ,the reason of progesterone deficiency in PCOS patients during the luteal phase is unknown [175]. Women with polycystic ovary syndrome (PCOS) need higher levels of progesterone to slow the frequency of gonadotropin releasing hormone pulse secretion [176]. Women who have had a history of miscarriage and are experiencing early pregnancy bleeding may benefit from vaginal progesterone therapy. Treatment with 400 mg of vaginal micronized progesterone twice day was linked to a higher incidence of live births [177]. In patients with PCOS, there are certain anomalies in progesterone synthesis that may be linked to a high risk of miscarriages [178]. **Prossnitz et al in (2007)** Found that ESTROGENS are released in considerable numbers exclusively by the ovaries in non-pregnant women, however small amounts are also secreted by the adrenal cortices. The placenta also secretes large amounts of ESTROGENS

during pregnancy [179]. High ESTROGEN levels, can develop in women with polycystic ovarian syndrome (PCOS). This disorder is characterized by a hormonal imbalance that can result in irregular periods, unwelcome hair growth, and acne [180]. The ESTROGEN dominance found in women with PCOS is caused by hormone imbalance. Environmental variables might also influence the outcome [181]. The levels of total testosterone and free testosterone were linked to the risk of PCOS [182]. Sometime testosterone levels may be normal in PCOS. Oral contraceptives reduce testosterone levels [183].

4.1.1 Age & Body Mass Index between groups

In this study, patients women with PCOS and control group were classified into two 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 45) years old . In our study, we found that the results of age (15-45) years, levels were increased, and the body mass index level almost equal in control and patients women with PCOS. Table (3.1). Because a woman's reproductive ability reduces with age due to decreased ovarian reserve, oocyte quality, and the increased occurrence of embryonic aneuploidy, age is a key factor impacting female fertility [184]. PCOS is the most commonly hormonal condition among women of reproductive age [185]. **Malizia et al in (2009)** In general, female fertility decreases as she gets older [186]. **Asamarai et al in (2009)** PCOS was associated with an increase in ovarian volume and follicle number from the age of 15 until menopause [187]. Each person's body mass index (BMI) was calculated using the formula weight (kg)/height (m^2), and the results were assessed to determine their obesity status. Not all PCOS women are obese, and not all PCOS women have an abnormal LH/FSH ratio, nor do they all have the hormonal and biochemical abnormalities associated

with the illness. According to other studies, a higher BMI always associated with a higher LH and a higher risk of menstrual disorders. Therefore, compared to obese female, non-obese women with PCOS had significantly higher blood LH levels [188].

4.2 The changes in fertility hormones between groups for age (15-29) years

In group 1 for age (15 to 29) years the results of hormones show that INHIBIN B and ACTIVIN A levels were decreased ,but LH, FSH, ESTROGENS, prolactin, progesterone and testosterone levels were increased in patients women , as shown in table (3.2). INHIBIN B levels are now thought to be the strongest predictor of reproductive potential. In fact, when it comes to determining reproductive potential, INHIBIN B outperforms follicle-stimulating hormone (FSH). There is a recognized negative association between INHIBIN B and FSH [189]. Premature ovarian failure affects 1% of all women and 0.1% of those under the age of 30 year. Secondary amenorrhea, infertility, and increased gonadotropin levels are all symptoms of this illness [190]. A similar study found that when the ovarian follicular reserve begins to decrease, serum INHIBIN B concentrations decrease [191]. Another study found that when comparing PCOS to normal people, follicular fluid INHIBIN B levels are lower [192]. ACTIVIN A and INHIBIN B are two closely related protein complexes with biological functions that are virtually diametrically opposed. ACTIVIN A contributes to the control of the menstrual cycle by enhancing FSH production and secretion. Many more activities of ACTIVIN A have been discovered, including functions in cell proliferation, differentiation, metabolism, homeostasis, immunological response, wound healing, and endocrine function are all important aspects of human health [193,194]. Women with polycystic ovary syndrome

have a three-fold increased risk of miscarriage compared to women without the disease [195]. ACTIVIN A promotes the anterior pituitary's production of FSH [196]. **Norman et al in (2001)** Suggested that the ACTIVIN A levels is altered in patients women with polycystic ovary syndrome in two groups.

Serum ACTIVIN A levels were considerably lower in PCOS patients than in controls [197]. **Shen et al in (2005)** In another study found that ACTIVIN A levels were shown to be lower in PCOS patients [198]. **Luisi et al in (2003)** Found that serum ACTIVIN A levels in miscarriage patients, have shown different results, with levels much lower in patients women [199]. The FSH and LH hormones are a contentious criteria for identifying a subset of miscarriages women with polycystic ovary syndrome and anomalies in the hypothalamic-pituitary-ovarian axis [200]. The high level of LH was detected in this study was clarified by **MecCartney et al in (2002)** who found PCOS women were shown to have a higher frequency and/or quantity of LH pulses, as well as an increase in LH secretory burst mass and a greater disorder in LH secretion.

According to one research, 75% of PCOS women had an increased LH level as a result of the elevated insulin levels that create the anomalies in the hypothalamic-pituitary-ovarian axis that cause PCOS [201]. In teenage and young women with polycystic ovaries, ultrasound is not a useful diagnostic tool. On ultrasonography, up to 70% of young women may have polycystic ovaries [202]. Age-specific interpretations of FSH concentrations might lead to more accurate ovarian function assessments [203]. The FSH level is the highest limit in patients women with polycystic ovary syndrome [204]. The presence of a high level of FSH or LH in the blood implies that there is a problem with the ovary. While

FSH or LH levels that are low or normal suggest a problem with the pituitary or hypothalamus [205]. PCOS is more common in younger women than in older women, possibly because to a physiological drop in the follicular cohort, which results in a normalized ovarian ultrasound appearance with age [206]. Several hormonal factors, as well as age, regulate prolactin production. Moderately high estrogen levels are linked to PCOS, which may promote prolactin production [207]. Recurrent miscarriages, which affects 2-5 percent of couples, is a severe concern [208]. Recurrent miscarriages are caused by a variety of endocrine variables, including increased prolactin [209]. As is well known, abnormally high levels of prolactin in the blood induce menstrual cycle problems and infertility, as well as repeated miscarriages, which impair pregnancy outcomes [210]. **Bahceci et al in (2003)** Suggested that Prolactin levels were shown to be greater in PCOS patients [211].

Women patients with PCOS generally have similar ESTROGEN levels higher testosterone hormone [212]. testosterone hormone is present normal or elevated levels in women with PCOS [213]. The presence of polycystic ovaries in patients causes a high level of testosterone, which has been proved by research and worldwide studies. The male hormone was the cause of secondary amenorrhea in patients owing to polycystic ovarian disease [214,215]. Patients with PCOS have greater testosterone levels in their blood [216]. Study by **Bruin et al in (2004)** increased testosterone output by the ovarian cells may increase the tiny number of potentially recruit follicles in the ovaries, and these follicles may cause not ovulating in patients with PCOS [217].The increase of testosterone hormone was in assent with the study of **Carmina et al in (2006)** [218]. Estrogen production changes quantitatively between patient women. The placenta is the major estrogen source , not the ovaries as in non-pregnant

women [219]. Due to a lack of ovulation, estrogen levels remain high. Constant estrogen exposure, can cause the endometrium to thicken abnormally, resulting in heavy and/or irregular bleeding or anovulation uterine bleed (periods without ovulation) [220]. To briefly reduce bleeding and stabilize the endometrial lining in women with menorrhagia ,estrogen in conjunction with progesterone may be prescribed [221]. In the plasma of a human female, only three estrogens are found in substantial amounts: -estradiol, ESTRONE, and ESTRIOL. -Estradiol is the most common estrogen released by the ovaries. ESTRONE is also produced in small amounts, although the majority of it is created in peripheral tissues from androgens secreted by the adrenal cortices and ovarian cells. ESTRIOL is a weak estrogen that is produced through oxidation of both estradiol and ESTRONE, with the conversion taking place mostly in the liver [222]. The results disagree with the study found that the ESTROGEN hormone level in PCOS women is low to normal [223]. Progesterone is released in significant amounts by the luteal phase in the non-pregnant female only during the latter half of each ovarian cycle. Large amounts of progesterone are also secreted by the placenta during pregnancy, especially after the fourth month of gestation [224]. Ovulation is intermittent in women with PCOS, shows the slight increase in ESTROGEN and progesterone levels seen in non-ovulating cycles [225]. **Chhabra et al in (2005)** suggested that women with PCOS need greater progesterone level [226,227].

4.2.1 Age & Body Mass Index between groups for age(15-29)years

In our study, found that age (15-29) years, levels were insignificantly increased in female with polycystic ovary syndrome, and the BMI level was insignificantly decreased in patients women.

4.3 The changes in fertility hormones between groups for age (30-45) years

In group 2 for age (30 to 45) years the results of hormones showed that INHIBIN B , ACTIVIN A, FSH, progesterone and testosterone levels were decreased ,but LH, ESTROGEN and prolactin levels were increased in patients women , as shown in table (3.3). Increased or decreased hormone levels that are greater or lower than normal levels might affect the ovarian system and lead to PCOS. As a result, health issues associated with advancing age may increase the chance of PCOS in women diagnosed with health problems such as obese, stroke and other conditions, and PCOS may exacerbate these conditions. As a result, early detection and treatment of PCOS patients are critical for reducing health risks in women. In our study, found that INHIBIN B and ACTIVIN A in two groups (15-29),(30-45) years, it was in the control group higher than patients women with PCOS. Study agrees with the study that found INHIBIN B secretion was shown to be declining in older women, indicating a decreased ovarian follicular pool [(228)]. Another study found that Follicular phase INHIBIN B levels were considerably lower in older females [(229)]. A similar study was found that the decrease blood ACTIVIN A levels (a glycoprotein that binds to ACTIVIN A and inhibits its function) are also found in PCOS patients [(230)].

The results of FSH show that hormone was decreased in PCOS women for age (30-45) when compared with the age (15-29) in which that hormone was increased in patients with PCOS. The changes in sex hormone levels were shown to be one of the most critical diagnostic

criteria for polycystic ovarian syndrome. The blood level of FSH was shown to be lower in women with this disease [231]. Other studies have found that PCOS can lower the release of FSH hormone [232]. Furthermore, testosterone may increase the number of FSH receptors in women with PCOS, resulting in a decrease in FSH concentration and an increase in LH serum levels [233]. The hormonal assay revealed that FSH was considerably lower in PCOS patients compared to healthy controls, the results agreement with **Begawy et al in (2010)** [234]. The results of LH, ESTROGEN and prolactin hormones was in these two groups (15-29),(30-45) years showed that the women with PCOS had higher than the control group. Previous research on LH and FSH serum level fluctuations in PCOS has shown that there is a link between increased LH and decreased FSH levels in PCOS [235,236]. **Jamil and colleagues in (2016)** found that the LH hormone were discovered to be significant predictors of PCOS diagnosis [237]. **Guyton and Hall in (2001)** found that increased estrogen release causes a reduction in FSH release in the pituitary, resulting in a negative feedback loop [238].

Goh & Ratnam in (1990) suggested that ESTROGEN high levels have also been linked to an increase in prolactin release [239]**. Sedighe et al in(2014)** some researchers agreed with this result, suggesting that blood prolactin levels in PCOS individuals are greater than usual [240]. **Szosland et al in (2015)** Many scholars disagreed, saying that serum prolactin levels in PCOS patients are normal and that the relation between PCOS and high serum prolactin levels is only accidental [241]. The results of progesterone and testosterone show that hormones was decreased in PCOS women for age (30-45)years when compared with the smaller age (15-29) years in which that hormones was increased in patients with PCOS. However, the earlier studies were show that

during the luteal phase, PCOS women had considerably lower progesterone concentrations than controls, according to the current study. Early pregnancy losses in PCOS women may be caused by decreased progesterone levels during the luteal phase [(242)]. Many researchers agreement with the study and found the mechanism by which LH induces recurrent miscarriages in PCOS women is unknown, the study provided more support for the negative effect of high LH in recurrent miscarriages in PCOS women. High LH levels were shown to have a negative relation with progesterone levels during the luteal phase in PCOS women , suggesting that low progesterone levels in older women may be linked to LH hyper secretion in this disease [(243)]. **Pinola et al in (2015)** found that the amount of testosterone in PCOS decreases with age [(244)]. Another study agreed with our study and suggested that the women with PCOS, their testosterone levels decrease as they become older [(245)].

4.3.1 Age & Body Mass Index between groups for age (30-45)years

In this study, found that age (30-45) years, levels of age were significantly increased in patient compared with control. This leads to an increase in body mass index in patient women (positive correlation). Table (3.3). In addition, both for control and PCOS patients, BMI in the age group (15-29) was lower than in the age group (30-45), although BMI in the control of age group (15-29) was higher than PCOS patients, and BMI in the age group (30-45) was lower than PCOS patients, and this study agreed with the study by **Shenta Ashwaq, Khansaa Saud, and Ali Al-Shawi in (2020)**. Another study by **Wild et al in (2000)** found that in a 31-year follow-up research, a PCOS group had significantly higher BMI values than a control group [(246)]. In the Rotterdam research, patients with PCOS had a higher BMI than age matched controls [(247)].

Most studies, but not all, support the assumption that PCOS are more overweight/obese at older ages compared with healthy controls.

4.4 Body Mass Index (BMI)

The BMI levels of non-pregnant women patients with PCOS showed that, the highest percentage of body mass index was for obese women with PCOS about 39%, and then for overweight women about 34%, while women with normal weight had the lowest body mass index for patients about 27% , as shown in figure (3.14). Several studies and research supported the result of the study, like as **Al-Tu'ma et al in (2015)** this is a common finding among women with the syndrome. Obesity and abdominal fat accumulation, which a high percentage of female suffer from, aggravate the physiological, hormonal, and metabolic symptoms of polycystic ovarian syndrome (PCOS) [(248)]. Another study found that the inability of insulin to operate properly is one of the reasons why people with PCOS gain weight or have difficulty losing weight. Obesity can lead to a variety of major health issues. Increased PCOS symptoms include irregular ovarian cycles, and anovulation. Endocrine and metabolic problems may be influenced by BMI. Obesity has been associated to an increase in number of miscarriages , cardiovascular and infertility risk in the development and progression of the syndrome [(249)]. According with World Health Organization (WHO, 2000), thin is defined as a BMI of less than 18.5 kg/m², normal weight is defined as a BMI of 18.5 to 24.9 kg/m², overweight is defined as a BMI of 25 to 29.9 kg/m², and obese is defined as a BMI of more than 30 kg/m² [(250)]. Obesity has been linked to a decrease in ovulatory rates, an increase in the frequency of miscarriages and possibly an increase in infertility associated with PCOS, according to a new study. Anxiety, melancholy, stress, and personal dissatisfaction, all of which are common in women with PCOS,

may be exacerbated by a change in body image as a result of weight increase [251].

4.4.1 The effect of body mass index (BMI) on biochemical parameters concentration in patients and control group

They were divided into three groups, those with a BMI. Normal weight was divided as group A (18.5-24.9 Kg/m²), overweight were classified as group B (25-29.9 Kg/m²), while those over(>30 Kg/m²) obese as group C. The current study showed that the levels of ACTIVIN A and INHIBIN B concentration with BMI, both hormones was higher in controls group than patients women with PCOS. In ACTIVIN A concentration , the BMI of the control group was higher in over weight when compared with normal weight and obese, while in the INHIBIN B concentration, the BMI of the control group was equal in each of the women with normal weight, over weight and obese , as shown in figure (3.15),(3.16). A study that agrees with our study, which found that the body mass index of ACTIVIN A was decreased in women with PCOS compared to healthy women [252]. The reality is that not all women with PCOS are overweight [253]. The diagnosis of PCOS is necessary because it indicates metabolic problems, possible cardiovascular problems, and, most importantly, it interferes directly with these patients' reproductive state [254]. INHIBIN B levels are inversely associated to BMI in polycystic ovarian syndrome. Suggesting that BMI may inhibit INHIBIN B release, and obesity reduces follicle health follicular production of INHIBIN B when compared to the control group [255].

When the women with PCOS and control group were subdivided according to their BMI, the concentration of testosterone and

progesterone in normal-weight women with PCOS were higher than in the control group, and healthy women overweight and obese women were higher compared to the patients, as shown in figure (3.17),(3.18). A study found that a little but significant percentage of women with PCOS have a normal or low BMI which may or may not even have symptoms like irregular menstruation [256]. A similar study by **Bellver, J., Rodríguez-Varela in (2022)** found that the patients who were overweight or obese had significantly lower serum progesterone levels than those who were underweight or normal weight [257]. When compared to obese women with PCOS, non-obese females with PCOS showed higher levels of testosterone [258]. Another study, on the other hand, indicated that testosterone levels are higher in overweight and obese women, and that disagreement with our study [259].

In this study, it was found that the body mass index of ESTROGEN and prolactin hormones in normal-weight and overweight women with PCOS was higher than in obese women compared to the control group, as seen in the figure (3.19),(3.20). **Randolph et al in (2011)** When compared to non-obese women, obese women had lower premenopausal ESTROGEN levels, which are measured on days 2–5 of a spontaneous menstrual cycle [260]. A study that agreed with our results shows that the prolactin level in PCOS women was greater in normal body weight women than overweight and obese women [261].

The normal BMI and obese with concentration of FSH hormone was higher in women with PCOS than the control group, and the results of levels of BMI with LH concentration in patients women higher than control, as shown in figure (3.21),(3.22). Some studies have found a higher of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in PCOS patients with normal weight than in obese PCOS patients

Dale et al in (1992) however, this result has not been found in other research **(Fulghesu et al in 1999; Moran et al in 2003)** [(262)]. Obesity is more common in women who have PCOS. Although increasing research shows that BMI adds significantly to the severity of many problems, like the risk of miscarriage, the relative impact of PCOS diagnosis and obesity in this group of women is still unknown [(263)].

4.5 The effect of the duration of miscarriage on biochemical parameters levels in patients women

In current study, found the relation between duration of miscarriage and concentration of biochemical parameters of patients women, and its effect on level of fertility hormones in one month, two months and three months or more. The results of the research show that the level of ACTIVIN A concentration for patients women with the duration of miscarriage, where the one month was more miscarriage than the three and two months, as shown in figure (3.23). The measurement of The levels of INHIBIN B, ESTROGENS and LH concentration in patients with duration of miscarriage, showed that the levels of these hormones were higher in one month when compared with two and three months, as shown in figure (3.24),(3.25),(3.26). The measurement of the levels of prolactin, progesterone, FSH and testosterone hormones with duration of miscarriage notice the more miscarriages in three months or more from pregnancy than one and two months from embryo age, as shown in figure (3.27-30) . Early miscarriage is described as a pregnancy loss that happens within the first three months of pregnancy (less than 12 weeks gestation) and affects 1-5% of pregnancies. Late miscarriage happens in the second trimester (12–24 weeks) and uncommon happen, affects in 1–2% of pregnancies [(264)]. A matching study showed that the first trimester failure is more common than the other months [(265)]. Females

with polycystic ovary syndrome are three times more likely than women without PCOS to miscarry in the first trimester of pregnancy [266]. Other studies, on the other hand, relate the rise to other factors like as obesity, advancing age, and the use of reproductive treatment [267,268].

In recent years, threatening miscarriages caused by environmental and societal causes have more common. During the first trimester, sex steroids such as progesterone and ESTROGEN, as well as prolactin and testosterone, regulate communication between the embryo and the mother. There are presently no reliable indicators that can be used to predict and evaluate first-trimester pregnancies with bleeding. The key indices used to identify and determine the prognosis in women facing an abortion danger are blood progesterone levels. In some cases, ESTROGEN levels are also used. Bed rest, luteal support, and combination ESTROGEN and progesterone supplements are the most common treatments for threatened miscarriage [269]. **Szekes Bartho et al in (2009)** showed that miscarriage during the first three months of pregnancy is associated with a decrease in progesterone concentration, which occurs in cases of recurrent miscarriages as a result of the mother's immune system rejecting the embryo [270]. In the second and third trimesters of pregnancy, the placenta is the primary producer of progesterone, so a problem with the placenta would result in a decrease in this hormone, while a problem with the luteal phase would result in a disruption in progesterone synthesis during the first trimester of pregnancy [271]. Miscarriages are linked to sex hormonal imbalance. The sex hormonal system is a complex orchestra of hormones that work a strict timetable. If any of these hormones are out of balance, it can have a negative effect on the entire circulation [272].

The embryo placenta is affected by low blood oxygen level, which causes an increase in the secretion of ACTIVIN A , from the placenta in late pregnancy. The ACTIVIN A hormone continues to increase during pregnancy until birth. The absence of internal uterine oxygen may also be indirectly reflected by this hormone. At the start of pregnancy, the amount of ACTIVIN A is checked because it may be used to diagnose trophoblast function deficiencies and regulate early pregnancy needs in the first trimester in women who have pregnancy-related bleeding that risks miscarriage. Cell division is controlled by ACTIVIN A, which also increases the secretion of follicle-stimulating hormone [(273)]. The reason for the high concentration of follicle-stimulating hormone is the effect of ACTIVIN A concentration in women with recurrent miscarriages, and this is consistent with what the researchers **Dipro&,Harris** said, who found the relation between the two hormones and their effects on the women body [(274)]. INHIBIN B concentrations were shown to be greater in embryo blood throughout the first trimester, according to research by **Muttukrishna et al in (2004)** [(275)]. The fact that decrease INHIBIN B concentrations indicate ovarian insufficiency in women who had missed miscarriages suggests that it may be possible to use INHIBIN B to predict the viability of an early pregnancy [(276)].

4.6 The effect of number of miscarriages on fertility hormones concentration in patients women

In this study, patients group were categorized according to the number of miscarriages into women with two, three or more miscarriages and its relation with the level of fertility hormones to find out the difference between the hormones. It was found that the concentration of ACTIVIN A, ESTROGEN, FSH, progesterone and testosterone

hormones with the number of miscarriages for patients women , that women with two recurrent miscarriages have a higher percentage than other women with the number of miscarriages of three or more, as shown in figures (3.31-35). While the measurement of the concentration of INHIBIN B, LH and prolactin hormones with the number of miscarriages, it was found that the level of hormones in women with three miscarriages or more is higher than in women with two recurrent miscarriages, as shown in figures (3.36-38). The most significant risk for miscarriage is a recent spontaneous miscarriage, according to a large body of research on recurrent miscarriage. Because a prior spontaneous miscarriage is the most important predictor of future spontaneous miscarriage, the result of a woman's first pregnancy has far-reaching significance for all future pregnancies [(277)]. Endocrine problems are thought to be responsible for around 8% to 12% of all occurrences of recurrent miscarriages [(278)]. Moreover, when a pregnancy is obtained, PCOS patients are more likely to suffer recurrent miscarriages [(279)]. Several research have investigated the correlation between PCOS and recurrent miscarriages in recent years. Women with recurrent miscarriages have been found to have higher rates of PCOS [(280)]. **Wang et al in (2001)** That found obesity was shown to be associated with an increased number and risk of recurrent miscarriage in PCOS patients [(281)]. According to some authors, insulin resistance, is a major role in understanding the link between obesity, PCOS, and recurrent miscarriages [(282)].

Several previous studies have found that women with lower levels of serum progesterone can lead to miscarriage [(283,284)]. However, increased ESTROGEN levels might cause miscarriage too [(285)]. This is consistent with our study that found low levels of progesterone in patients

women for age 30 to 45 years, and higher levels of ESTROGEN in patients for both age groups, and this explains the relation of hormones and their effect on the number of recurrent miscarriages. No treatment to stop miscarriages in women at risk of miscarriage. There is some evidence that progesterone may enhance the number of live births among women who experience recurrent miscarriages [(286)]. It was discovered that use vaginal progesterone enhanced the live birth rate and decreased the miscarriage rate for women who had experienced recurrent miscarriages. The group of women with a history of three or more miscarriages had a greater live birth rate than the group of women with a history of two or more losses [(287)]. According to a study, found the high in ACTIVIN A during the first two weeks of pregnancy can be used to predict how the pregnancy would end out. The concentration of ACTIVIN A appears to be lower in pregnancies that are doomed to miscarriage than in pregnancies that end in birth [(288)]. In fact, INHIBIN B levels never rise to high levels throughout pregnancy, and their highest levels at birth are similar to their peak mid cycle values a few days after ovulation [(289)].The hormone prolactin is important for female reproduction and is crucial for maintaining the health of the luteal phase and progesterone release. In women who experienced recurrent miscarriages, prolactin levels were linked to a higher risk of miscarriage in future pregnancies [(290)]. In comparison to fertile controls, ovarian reserve was evaluated in women who experience recurrent miscarriages. Women who often miscarried had higher FSH levels [(291)]. While high levels of luteinizing hormone in the early to mid-follicular phase have been linked to a higher risk of miscarriages in women with and without PCOS, as well as Increased testosterone levels are linked to a decrease in uterine growth during the luteal phase and are thought to be responsible in recurrent miscarriages.

Women who suffer from recurrent miscarriages had greater testosterone levels than fertile controls [292].

4.7 The effect of last miscarriages date on biochemical parameter concentration

In this study, the group of women patients was divided according to the last miscarriages date into three groups, the first group the last miscarriage within (1-4) months, the second group from (5-9) months, and the third group more than (9months -2years) and the relation with the concentration of fertility hormones for the purpose of knowing their effect on the level of these hormones. The study found that the level of concentration of ACTIVIN A, FSH, progesterone and testosterone hormones was higher percentage in first group(1-4 months) for the last miscarriages date for patients women when compared with the second group (5-9 months)and third group (over 9 months - 2 years), as shown in figure (3.39-42), but the level of the INHIBIN B concentration with the last miscarriages date of patients ,showed insignificantly decreased in the second group (5-9 months) when compared with the first group (1-4 months), as shown in figure (3.43). While the level of concentration of LH, ESTROGEN and prolactin hormones was higher percentage in second group(5-9months) for the last miscarriages date for patients women when compared with the first group (1-4 months)and third group (over 9 months - 2 years), as shown in figure (3.44-46). Maternal age has been shown to have a significant impact on pregnancy outcomes [293]. Not only does age have an impact on future pregnancy outcomes, but it also has an impact on fertility [294,295]. Mostly in terms of underlying fertility It not only decreases with maternal age, but it also differs by age group [296]. Increasing pregnancy period after a miscarriage, on the other hand, tends to enhance birth outcomes in the subsequent pregnancy. In order to

improve results, a world health organization (WHO) expert consultation on birth spacing suggests deferring the next pregnancy for at least 6 months after a miscarriage. This advice was based on the findings of a single big Latin American study, which found that miscarriage–pregnancy durations fewer than 6 months were linked to a higher risk of preterm birth [(297)]. Study in California found that revealed no evidence of negative related with pregnancies soon after a miscarriage [(298)]. Another study found that, in the setting of recurrent miscarriages, fertility is highest when the delay between pregnancies is at least one per year. This suggests that the appropriate waiting period for a pregnancy is around 6–9 months; a conclusion that has been somewhere else in relation to the time to pregnancy following a miscarriage [(299)]. After the lady has experienced many pregnancies. Our finding suggest that when the waiting period reaches around 6 to 9 months, there should be knowledge of masculinity factor. Use of nicotine has been linked to an increased risk of miscarriage and infertility [(300)].

Two studies on hormone levels in the menstrual cycle following spontaneous miscarriage have been conducted, Low levels of follicle-stimulating hormone and luteinizing hormone (LH) were associated with reduced pituitary function, according to one theory [(301)]. the second suggested decreased luteal estrogen and progesterone levels as well as lower LH levels [(302)]. Reduced fertility in the post-last miscarriage cycle would result from these hormonal changes [(303)]. Women's fertility decrease gradually but significantly starting around age 32 and more quickly after age 37, this is mainly due to a decrease in egg production in conjunction with a slight rise in circulating levels of follicle-stimulating hormone and a decrease in INHIBIN B concentrations [(304,305)]. The decrease of INHIBIN B levels are a more accurate late predictor of

decreased follicle number. As a result, INHIBIN B levels are not suggested to be used as a marker of the ovarian poor response to ovarian stimulation or as a useful biomarker of ovarian reserve [306]. Unsurprisingly, pregnancy loss results in a rapid decline in the levels of maternal serum ACTIVIN A, often even before clinical symptoms show [307]. But a recent study in a different group found that these indicators were useless at diagnosing imminent miscarriage in asymptomatic women [308].

CHAPTER FIVE

CONCLUSIONS

&

RECOMMENDATIONS

5.1 Conclusions

In summary, this study proved the following:

1. Women who are overweight and obesity are more likely to have recurrent miscarriages.
2. Variation in the levels of several hormones such as LH, FSH, ESTROGEN, ACTIVIN A, INHIBIN B, prolactin, progesterone and testosterone is an indicator of the possibility of recurrent miscarriages.
3. Pregnant women after treatment of PCOS are more likely to have recurrent miscarriages than other women.
4. BMI for women with PCOS was the highest in obese women about 39%, overweight women about 34%, and 27% of normal-weight women, and this shows the relation of obese with recurrent miscarriages.
5. Decreased levels of both INHIBIN B and ACTIVIN A hormones for all age groups (15-45) years of women of childbearing age and their exposure to recurrent miscarriages. Measurement of these variables is suggested by their adoption to predict miscarriage or not.
6. There is an increase in the number of miscarriages in patients women with PCOS, the old miscarriage is the biggest indicator of a later miscarriage in the future.
7. It is best to space between births at least once a year, as the appropriate waiting period for pregnancy ranges from 6-9 months to avoid recurrent miscarriages.

5.2 Recommendations

1. Conducting measurements of each of the sex hormones in pregnant women for the purpose of making sure of miscarriage or not, especially in the first three months of pregnancy.
2. Adopting the measurement of both the INHIBIN B and ACTIVIN A hormones in order to know the possibility of miscarriage in pregnant women.
3. Recommending weight loss for women who want to get pregnant to increase the chance of completing the pregnancy without miscarriage.
4. Emphasis on the events of pregnancy at a young age for women and avoiding the desire to become pregnant among older women, that associated with the occurrence of risk of miscarriage.
5. The necessity of treating women suffering from PCOS to ensure a safe pregnancy and to avoid recurrent miscarriages.

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الخلاصة

صممت هذه الدراسة لمعرفة العلاقة بين الأسقاط المتكرر وهرمونات الخصوبة لدى النساء مع وجود بعض الأسباب الرئيسية للأسقاط مثل متلازمة تكيس المبايض والسمنة وعمر الأم.
اشتملت الدراسة على 90 امرأه تتراوح أعمارهن بين (15-45) سنة ، تم تقسيمهن إلى 50 امرأه مصابة بمتلازمة تكيس المبايض وتعانين من الأسقاط المتكرر و 40 امرأة كمجموعة تحكم صحية. أخذت العينات من مستشفى النسائية والتوليد التعليمي في كربلاء ومن العيادات الخارجية النسائية خلال الفترة من 1/10/2021 (تشرين الأول) إلى 29/9/2022 (سبتمبر) حيث تم قياس هرمونات الخصوبةFSH وLH و ESTROGEN وProlactin و Progesterone و Testosterone بواسطة تقنية ELISA وباستخدام جهاز CL-900i من شركة mindray في الصين ، وقياس هرمونات INHIBIN B و ACTIVIN A بواسطة تقنية ELISA من BioTek في الولايات المتحدة الأمريكية. توصلت الدراسة في نتائج التحليل الإحصائي أن هناك فرقًا معنويًا في ACTIVIN A و INHIBIN B و FSH و LH و ESTROGEN و Prolactin و Progesterone و Testosterone مقارنة بمجموعة التحكم ، حيث لاحظنا انخفاضًا في مستوى الهرمونات INHIBIN B و ACTIVIN A في مصل مرضى النساء المصابات بمتلازمة تكيس المبايض في جميع الفئات العمرية من (15-45) سنة مقارنة بمجموعة التحكم ، وزيادة في مستوى FSH و LH و ESTROGEN و Prolactin و Progesterone و Testosterone لدى النساء المرضى لعمر (15-29) سنة مقارنة بالمجموعة الضابطة ، بينما لاحظنا انخفاضاً في مستوى بعض الهرمونات لدى النساء الأكبر سناً من (30-45) سنة ، مثل Progesterone وFSH و Testosteroneمقارنة بمجموعة التحكم الصحية. كما أظهرت نتائج الدراسة أن النساء اللواتي يعانين من الأسقاط المتكرر بسبب متلازمة تكيس المبايض كان لديهن مؤشر كتلة جسم أعلى لدى النساء البدينات بنسبة 39%، مقارنة بالنساء ذوات الوزن الزائد بنسبة 34%، و النساء ذوات الوزن الطبيعي بنسبة 27%. وتشير ايضا الى وجود اختلاف في مستويات تركيز هرمونات الخصوبة مع مدة الاسقاط وعدد حالات الأسقاط المتكررة وتاريخ آخر اسقاط لدى النساء المرضى مقارنة بالمجموعة الضابطة.

جامعة كربلاء

دراسة هرمونات الخصوبة في النساء التي تعاني من الأسقاط المتكرر في محافظة كربلاء

رسالة

مقدمة الى كلية التربية للعلوم الصرفة/جامعة كربلاء, كجزء من متطلبات الحصول على درجة الماجستير في الكيمياء

من قبل

اسيل احسان محمود الموسوي

بأشراف

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