Summary

This study was constructed to discuss an issue regarding infertility cases that was attributed to hyperprolactinemia. About 150 blood samples were collected from women suffering hyperprolactinemia and 50 from normal subjects served as control for comparison. Test subjects were divided into three age groups: 20-30 years old, 31-40 years old and 41-50 years old. Tests of fertility hormones luteinizing hormone, follicle stimulating hormone and prolactin were performed for all study groups. It was found that there was a significant $P<0.05$ difference in hormone concentration for patients when compared with control. Luteinizing and Follicle stimulating hormone recorded a significant decrease while prolactin recorded a significant increase when compared with control. RNA isolation from serum was possible due to high expression of prolactin gene in study groups since an average concentration of 300 ng was obtained from serum.

At molecular level analysis using three specific primers designed for this study, showed that there is an aberration at the expression level of RNA in some of hyperprolactinemia patients while prolactin receptors were normal. It was concluded that in all patients feedback inhibition mechanism that controls prolactin level, was disrupted in addition some of patients studied were candidates for breast cancer as was reviewed from family history and most of hyperprolactinemia cases for subjects studied were attributed to hyper expression of prolactin gene.

Protein analysis of blood from patients showed that a significant increase in albumin, this was regarded to increased level of prolactin in the blood and this protein function as a carrier for hormones.

Acknowledgment

Praise to Allah the first cause of all causes, the glorious creator of the universe, and praise upon Mohammad his Prophet and upon his Family.

I would like to express my deepest gratitude and faithful thanks to my supervisor Dr. Rehab Subhi Ramadhan for her support, encouragement, and useful advices that she provided during this research.

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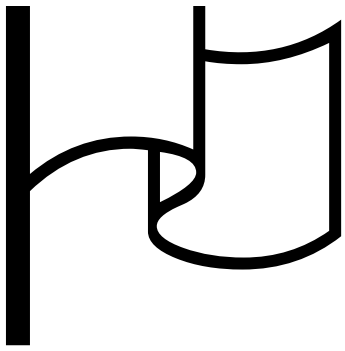
My thanks and gratitude to my dear friends Mrs. Sammara, K. Ibrahim, Estabraq, A. Mahdi, Sarah T. Kadhim and Motasm Yunis.

Still one last word to say, my praise to the merciful God that he heals all patients with this disease, and all other diseases and ends their suffering with his mercy.

Noor



قُل كلٌ يعملُ على شاكلتهِ فربكمُ اعلمُ

بمنْ هوَ أهدى سبيلًا _ ويسألونكَ عنِ

الروحِ قلّ الروحُ منّ أمِر ربي وما أُوتيتمُ

منَ العلمِ إلّا قليلا_

صدق الله العظيم

[سورة الإسراء – الآيات ٨٤ و ٨٥]

Appendix -1-

Case profile

Name	
Age	
Address	
Occupation	
Material Status	
Education	
No. of children	
Time of disease diagnosis	
Any more symptoms	
Nutrition	
Medication	

Appendix -2-

No:

Name:

Age:

Fertility hormones

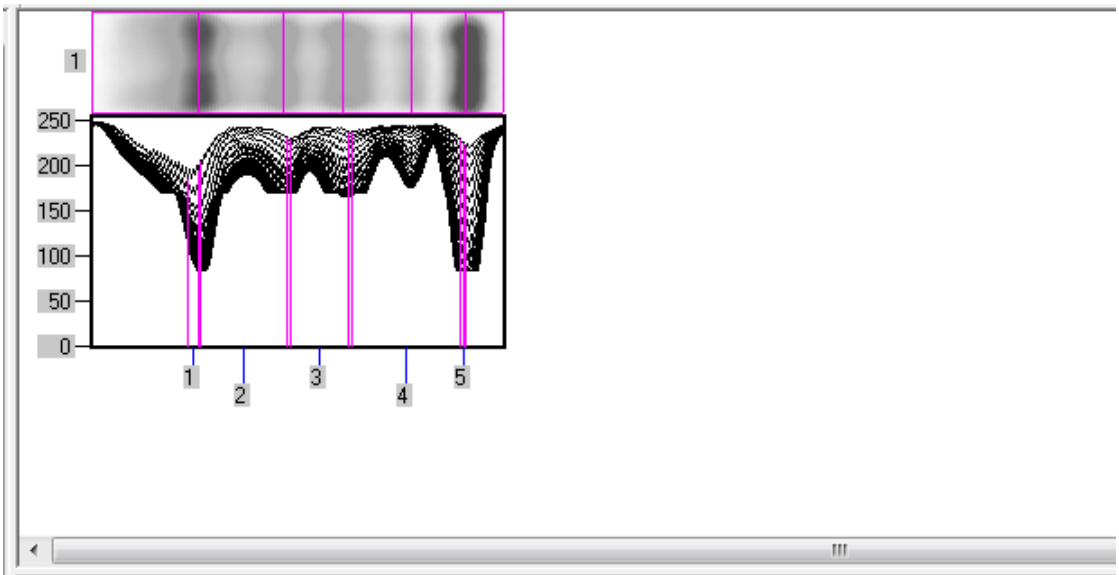
Prolactin	(ng/ml)
FSH	(ng/ml)
LH	(ng/ml)

Appendix -3-

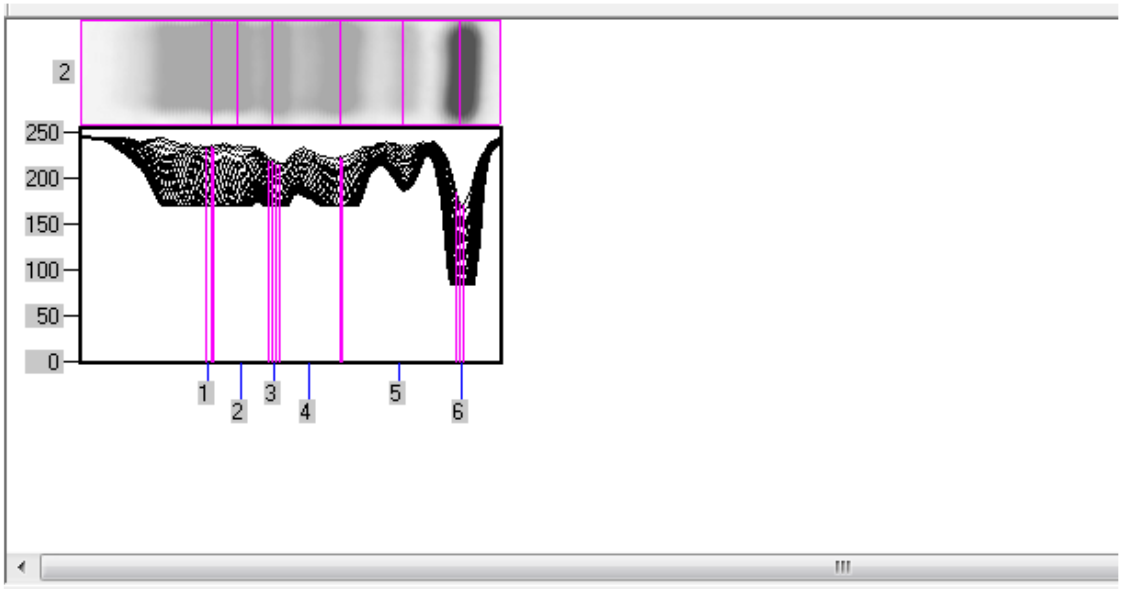
Governorate	Hospital name	hyperprolactinemic No. of patients
Baghdad	AL- Yarmok Teaching Hospital	100
	AL- Kindey Teaching Hospital	30
	St. Rafael hospital	20

Appendix -4-

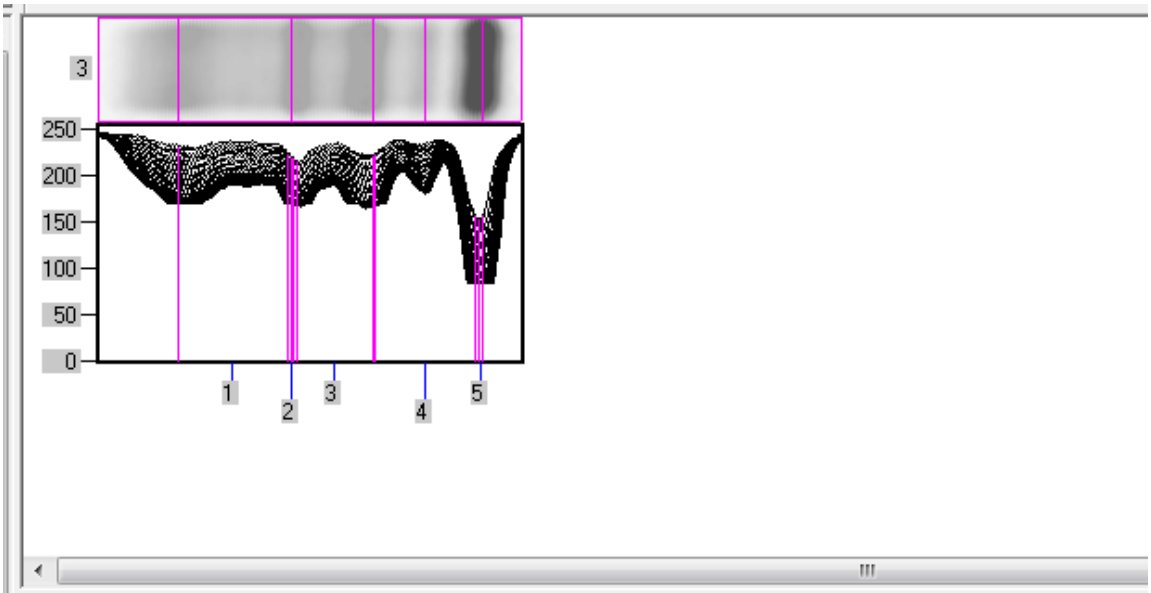
Protein electrophoresis analysis



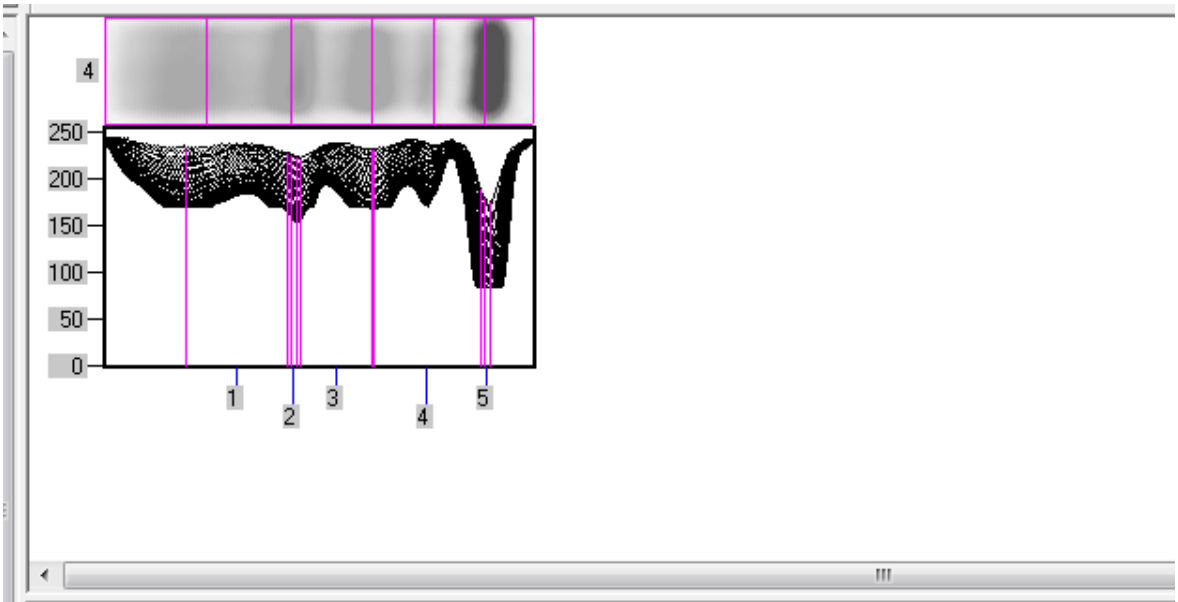
Number	Volume	Height	Area	------	M.W.	Number	Volume	Height	Area	------	M.W.
1	46532	200	399			4	682584	246	3477		
2	492278	242	2793			5	11398	226	114		
3	354698	242	1881								



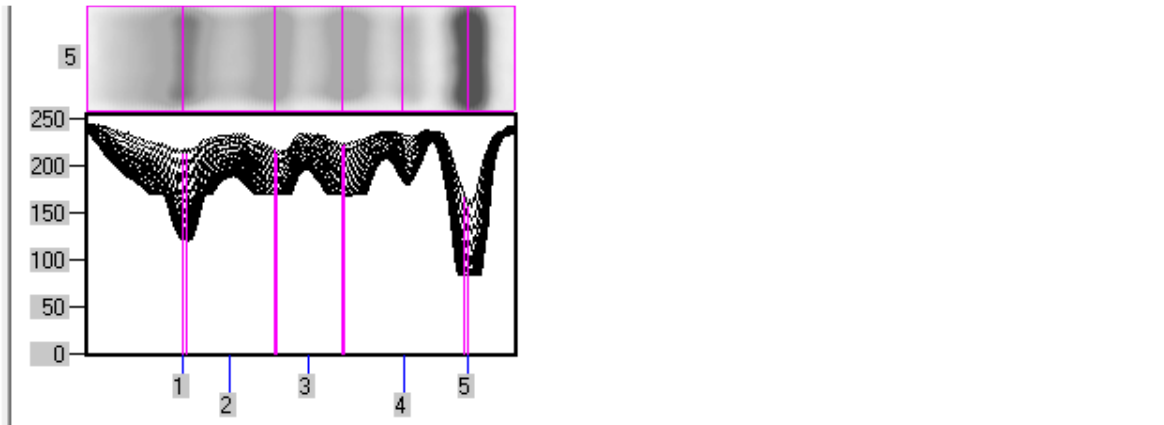
Number	Volume	Height	Area	------	M.W.	Number	Volume	Height	Area	------	M.W.
1	41114	236	232			4	359280	234	1972		
2	322518	238	1798			5	714494	240	3654		
3	30524	220	174			6	16442	174	174		



Number	Volume	Height	Area	------	M.W.	Number	Volume	Height	Area	------	M.W.
1	663984	238	3420			4	588914	238	3135		
2	19998	220	114			5	15542	158	171		
3	443672	236	2394								



Number	Volume	Height	Area	------	M.W.	Number	Volume	Height	Area	------	M.W.
1	600996	238	3190			4	649302	242	3364		
2	40140	226	232			5	22154	180	232		
3	427324	238	2262								



Number	Volume	Height	Area	------	M.W.	Number	Volume	Height	Area	------	M.W.
1	7902	214	57			4	706772	238	3705		
2	495864	234	2736			5	5098	158	57		
3	386732	234	2052								

1. Introduction and Literatures Review.

1.1. Introduction.

Prolactin (PRL) is a hormone secreted by the pituitary gland which is located at the base of the brain, the principal function of prolactin is the stimulation of milk production by the mammary glands of females after the birth of a baby. Normally, it is present in small amounts throughout the bloodstream typically 10-28µg/L in females and 5-10µg/L in males kept under control by another hormone called a prolactin inhibiting hormone (dopamine). The chemical composition of prolactin is protein undergoes several post-translational modifications that include polymerization, proteolytic cleavage, phosphorylation and glycosylation, these modifications impact prolactin stability, half-life, receptor binding and biological activity (Jameel, 2010).

Prolactin secretion may increase mildly with sleep, stress, coitus, exercise, nipple stimulation, ingestion of certain foods, and pregnancy. If the female's prolactin level is elevated the first time. A second sample should be checked when she is fasting and non-stressed. Confirmed elevation of prolactin need to be evaluated (American Society for Reproductive Medicine, 2009).

Hyperprolactinemia is a condition in which excess prolactin circulates in the bloodstream of non pregnant females and in males, it is probably one of the most common endocrine disorders that related to pituitary function. In females, hyperprolactinemia results in a variety of reproductive dysfunctions including a decline in the body’s production of progesterone during the luteal phase after ovulation, irregular ovulation, menstruation, and absence of menstruation. Also vaginal dryness making sex pain full and galactorrhea. In males, hyperprolactinemia can lead to reduce body hair, muscle, libido or impotence and cause inefficient sperm production and infertility (Colao, 2004).

Clinically significant elevation of prolactin levels may cause infertility in several different ways: first, excess prolactin may stop females from ovulating, If this occurs women's menstrual cycles will stop. Second, in less severe cases high prolactin levels may only disrupt ovulation once in a while this would result in intermittent ovulation or ovulation that takes a long time to occur so that, females in this category may experience infrequent or irregular periods. Third, females with the mildest cases involving high prolactin levels may ovulate regularly but not produce enough progesterone hormone after ovulation, this is known as a luteal phase defect. Deficiency in the amount of progesterone produced after ovulation may result in uterine lining that is less able to have an embryo implanted. Some of females with this problem may see their period come a short time after ovulation (Shibli-Rahhal and Schlechte, 2011).

Hyperprolactinemia is relatively common in females during reproductive age with stop menstruation period and have low follicle stimulating hormone (FSH) levels up to a third have hyperprolactinemia (Melmed, 2011). this study was suggested to fulfill the aim of :

- Early detection of prolactin hormone elevation in women because of its direct effect on fertility.

1.2. Literatures Review.

1.2.1. The endocrine system.

The endocrine system is an integrated system consists of several glands, hormones and scattered hormone secreting cells. It is an instrumental in regulating metabolism, growth, development, reproduction, also it plays a role in mood determining (Kester *et al*., 2004). The endocrine system and nervous system are interacted to maintain homeostasis. The nervous system acts faster and more locally than the endocrine system, however, the effects of the endocrine system are generally prolonged as well as the two systems are physically linked by neurosecretory cells, which are neurons that secrete hormones. The nervous system uses nerves to conduct information, whereas the endocrine system mainly uses blood vessels as information channels (Lewis *et al*., 2007).

Hormones are metabolically produced chemical substances released by non-neural endocrine cells or by neurons. It exerts regulatory influences on the function of other distant cells reached via the blood. The blood carries the hormones to the target cells that contain specific receptor proteins for the hormones, which therefore can respond in a specific fashion to them. Hormones can be classified according to their chemical structure into steroids, amines, polypeptides and glycoproteins (Hill *et al*., 2004).

In terms of their actions in target cells the hormones can be divided into those that are polar and therefore water soluble and those that are nonpolar, and thus insoluble in water. Polar hormones cannot cross cell membranes, but instead it bind to one of the hundreds of receptors on the target cell resulting in activation of a coupling protein (G

protein). This protein activates another membrane associated enzyme called adenyl cyclase that catalyzes conversion of ATP into cAMP which in turn activates specific enzymes within the cell. The nonpolar hormones are lipid soluble so they can easily cross through the cell membranes of the target cells and bind to a receptor in the nucleus resulting in the stimulation of particular genes (Fox, 2006).

The major endocrine glands are the pineal, pituitary, thyroid, thymus, adrenal, pancreas, ovary and testes as shown in figure (1.1). Endocrine glands are in general characterized by their ductless nature, their vascularity and usually the presence of intracellular vacuoles or granules storing their hormones. In contrast exocrine glands have ducts and tend to be less vascular such as salivary, sweat, and digestive glands (Console *et al*., 1995).

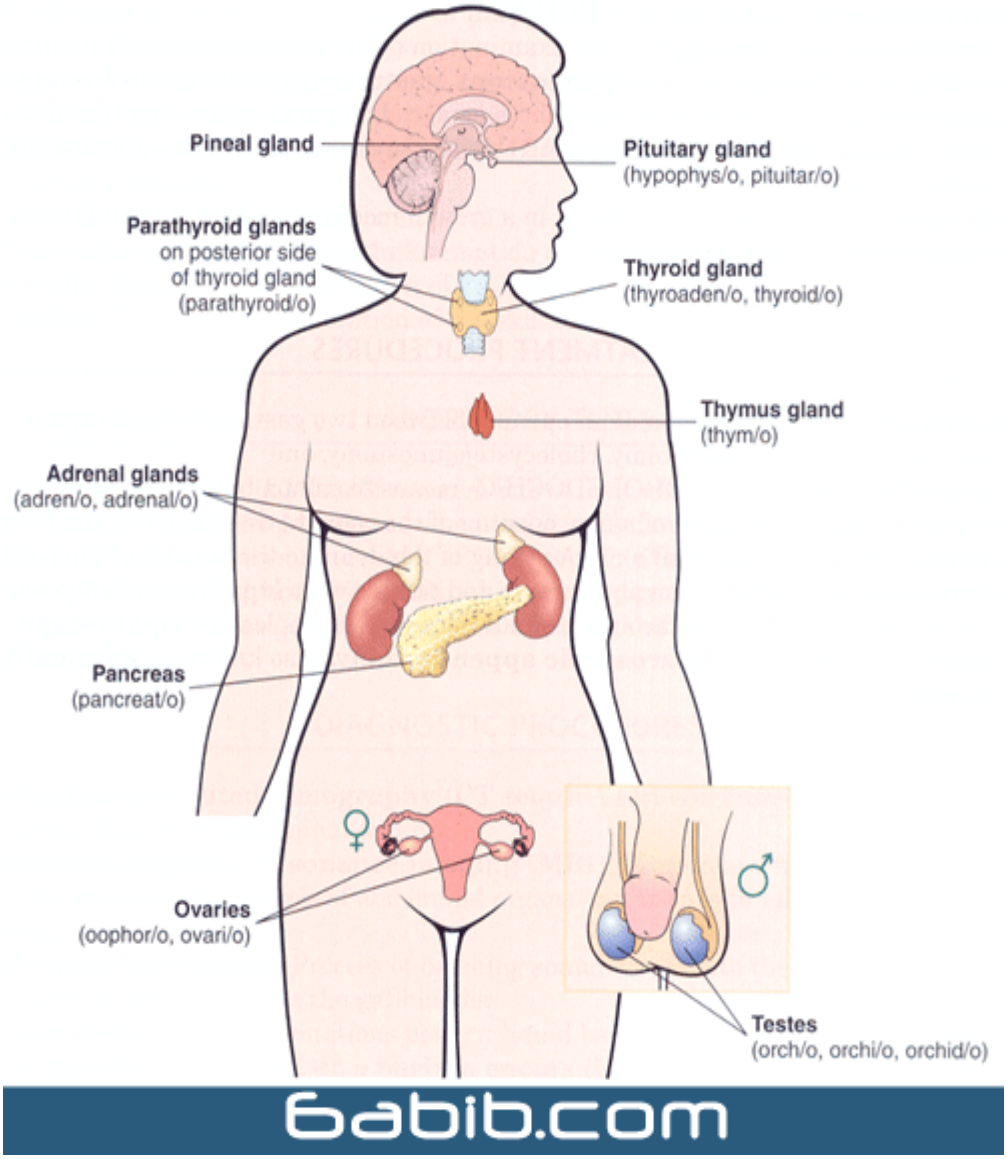


Figure (1.1) Major endocrine glands. (Mārcia *et al*., 2008).

1.2.2. The pituitary gland.

The pituitary gland is an endocrine gland that located at the base of the brain. Its size roughly 1.3 cm in diameter and it is attached to the hypothalamus by a stalklike structure called the infundibulum. The pituitary gland is structurally and functionally divided into an anterior lobe, or adenohypophysis, and a posterior lobe called the neurohypophysis. The posterior pituitary stores and releases hormones that are actually produced by the hypothalamus,

whereas the anterior pituitary produces and secretes its own hormone (Page, 1994; Tucker, 1999 and Greaves, 2007). The hormones secreted by the anterior pituitary are called trophic hormones, the term trophic means "feed" because high concentrations of the anterior pituitary hormones cause their target organs to hypertrophy, while low levels cause their target organs to atrophy. The hormones of the anterior pituitary include :

- Growth hormone (GH) promotes growth and development of all tissues by increasing proteins synthesis and cells division rates.
- Thyroid stimulating hormone (TSH) stimulates the thyroid gland to produce and secrete thyroxine (tetraiodothyronine, or T4) and triiodothyronine (T3).
- Adrenocorticotropic hormone (ACTH) stimulates the adrenal cortex to secrete the glucocorticoids, such as hydrocortisone (cortisol).
- Follicle stimulating hormone (FSH) is a glycoprotein with a molecular weight of approximately 3000 dalton . FSH is secreted by the basophilic cells of the anterior pituitary. It is responsible for the proliferation of the follicular cell, for the development of the graafian follicle and for ovum maturation.
- Luteinizing hormone (LH) in females, stimulates the ovulation and conversion of the ovulated ovarian follicle into an endocrine structure called the corpus luteum. In males, LH is sometimes called interstitial cell stimulating hormone (ICSH), it stimulates the secretion of male sex hormones (mainly testosterone) from the interstitail cells (leyding cells) in the testes.
- Prolactin (PRL) hormone is secreted in both males and females, its best known function is the stimulation of milk production by the

mammary glands of women after the birth of baby (Kaushansky, 2006).

1.2.3. Prolactin hormone.

1.2.3.1. Discovery of prolactin.

Stricker and Grueter in (1928) were discovered prolactin as a distinct hormone with lactogenic activity secreted by the anterior pituitary. Because of prolactin is closely related to growth hormone, human growth hormone binds to prolactin receptors and has a potent lactogenic activity so that, it was not discovered until the early 1970's as a separate entity (Lewis *et al*., 1971 and Hwang *et al*., 1972). The hormone being purified and given the name prolactin based on the fact that an extract of bovine pituitary gland would cause growth of the crop sac and promote the elaboration of crop milk in pigeons(Riddle and Braucher, 1931 and Riddle *et al*., 1932). Cooke and his colleagues in (1981) were discovered the entire amino acids sequence of prolactin, including 28 residues signal peptide, from the nucleotide sequence of human cDNA. Furthermore, the extrapituitary production of prolactin was first detected in decidualized endometrail cells (Riddle *et al.* , 1963) and subsequently in many other reproductive tissues, immune cells and brain.

1.2.3.2. Functions of prolactin.

Prolactin (PRL) is a polypeptide hormone that is synthesized and secreted by specialized cells of the anterior pituitary gland, lactotroph (Fitzgerald and Dinan, 2008). The main function of prolactin is stimulation of breast development and production of milk (Horseman, 1999). However, more than 300 distinct biological activities have been attributed to prolactin, including salt and water balance, cell growth and proliferation (Freeman *et al*., 2000). It also has effects on the

hypothalamo-pituitary-gonadal axis and can inhibit pulsatile gonadotropin releasing hormone secretion from the hypothalamus and alter the activity of certain steroidogenic enzymes. In females, excess prolactin secretion is associated with infertility and menstrual irregularity or even complete amenorrhea. In males, it causes decreased testosterone level and sperm production. In addition, excess prolactin can cause galactorrhea (inappropriate milk production) in females and gynecomastia "breast development" in males (Melmed *et al*., 2011).

Prolactin is synthesized at many extrapituitary sites particularly in reproductive organs, immune cells, and brain (Ben-Jonathan *et al.*, 2008) therefore, it is clear that prolactin can act as a local paracrine and autocrine factor in diverse tissues and cells (Yu-lee, 1997 and Bachelot, 2007). At the cellular level, prolactin regulates the survival, growth, differentiation, and activation state of target cells. At the physiological level, the effects of prolactin are reproduction, immune function, water electrolyte balance (osmoregulation), stress adaptation and metabolism. Prolactin in human serum has been classified into three main classes on the basis of molecular mass: monomeric PRL, big PRL and big-big PRL (bb-PRL) called macroprolactin with a molecular mass of 23 killodalton, 50-60 kD and 150-170 KD respectively (Baban *et al*., 2008). Prolactin secretion is regulated by a dual hypothalamic inhibitory and stimulatory system and can regulate its own secretion through a short loop feedback. Prolactin hypothalamic control is unique in that the predominant hypothalamic influence is inhibitory whereas for all other hormones the predominant influence is stimulatory. Thus, damage to the hypothalamic control causes increased prolactin secretion rather than decreased secretion, as seen with all other anterior pituitary hormones (Nussey and Whitehead, 2001). Dopamine is the main

neurohormone inhibiting prolactin secretion (Holt, 2008), while thyrotrophin releasing hormone has a potent stimulatory action on prolactin release, other factors including pregnancy, lactation (suckling), estrogen, dopamine antagonists, sleep and stress can also stimulate prolactin secretion (American Society for Reproductive Medicine, 2009).

During pregnancy, the high circulating concentrations of estrogen inhibit secretion and action of prolactin. This inhibition is accomplished by the stimulation of prolactin inhibiting hormone (dopamine) secreted from the hypothalamus (Fitzgerald and Dinan, 2008). After parturition, declining levels of estrogen are accompanied by an increase in the secretion of prolactin (Serri and Ezzat, 2004). Suckling helps to maintain high levels of prolactin secretion via a neuroendocrine reflex. When the sensory endings in the breast activated by the stimulus of suckling which relay impulses to the hypothalamus and inhibit the secretion of prolactin inhibiting hormone. Suckling thus results in the reflex secretion of high levels of prolactin that promotes milk production, as well as the stimulus of suckling results in the secretion of the oxytocin from the posterior pituitary which results in milk let down (Soka *et al*., 2011).

1.2.3.3. Prolactin gene.

The gene encoding prolactin is unique, in human its located on chromosome 6 and is composed of five exons and four introns for an overall length of 10 kilobase (Kb). The transcription of the prolactin gene is regulated by two independent promoter regions, the proximal 5,000 base pairs (bp) region directs pituitary specific expression, while a more upstream promoter region is responsible for extrapituitary expression (Noh *et al.*, 2012). Transcriptional regulation of prolactin

gene has been studied quite extensively as well the silico bioinformatics used to analyze the promoter region of prolactin gene (2000 bp upstream ATG start codon). Upon analysis of the prolactin gene *HOX- 1.3* and *XFD-2* transcription binding sites are found. They are the most specific transcription factor elements to master regulate the gene expression (Berwaer *et al*., 1994 and Featherstone *et al.,* 2012).

HOX-1.3 the homeobox genes are a superfamily of genes encoding transcription factors that regulate developmental processes such as body patterning and organogenesis (Krumlauf, 1994). The mammalian *HOX* gene complex consist of 39 genes that are located on four linkage groups (clusters) dispersed over four chromosomes (*HOXA, HOXB, HOXC and HOXD*). HOX-1.3 is a transcriptional regulator of many target genes, including p53 and the progesterone receptor. HOX1.3 is a transactivator of p53 and may affect the response of breast cancer cells to DNA damage (Aubin *et al*.,1997).

XFD-2 the recently described XFD (Xenopus fork domain related) multigene family in the frog *Xenopus laevis* that contain this DNA binding domain (Shawn *et al.,* 1988). Fork head proteins play an important role in embryonic pattern formation, regulation of tissue specific gene expression and tumor formation (El-Hodiri *et al*., 2001). These proteins contain a highly conserved 11 amino acid long DNA binding domain that was originally identified in Drosophila mutant fork head (Bulow *et al.,* 2010). Because of their structure, these proteins are also referred to as winged helix proteins. They bind DNA as monomers and can act as transcriptional activators or repressors (Kaestner *et al*., 2000).

The human prolactin mRNA is 914 nucleotides long and contains a 681 nucleotide open reading frame encoding the prolactin

prohormone of 227 amino acids. The 28 amino acids signal peptide is cleaved and the mature human prolactin is formed (Binart *et al.*, 2010). The mature human prolactin is composed of 199 amino acids with three intramolecular disulfide bonds between six cysteine residues (Fitzgerald and Dinan, 2008).

1.2.3.4. Prolactin Receptor (PRL-R).

The prolactin receptor is a single membrane bound protein that belongs to class I of the cytokine receptor superfamily (Bazan, 1990a; Bazan, 1990b; Kelly *et al*., 1991 and Lai *et al*., 1997). Prolactin and growth hormone receptors share several structural and functional features despite their low (30%) sequence homology (Goffin *et al.*, 1988; Goffin and Kelly, 1996). Each receptor consists of an extracellular, short transmembrane domain and a variable intracellular domain (ICD) that mediates signaling (Acs *et al*., 1993).

Numerous prolactin-R isoforms have been described in different tissues (Davis and Linzer, 1989; Ali *et al*., 1991) and these isoforms are results of transcription starting at alternative initiation sites of the different prolactin-R promoters, as well as alternative splicing of non-coding and coding exon transcripts (Hu and Dufau, 1991). Although the isoforms vary in the length and composition of their cytoplasmic domains, their extracellular domains are identical (1998; Kelly *et al*., 1991; Lesueur *et al*., 1991 and Bole-feysot *et al*., 1998). In addition to the membrane bound receptors, soluble prolactin binding proteins were also described in mammary epithelial cells (Berthon *et al.*,1987) and milk. These soluble forms contain 206 NH2-terminal amino acids of extracellular domain the prolactin-R (Berwear *et al*., 1994). The soluble prolactin binding proteins are also products of the same prolactin-R gene, but it is still uncertain whether they are results of alternative

splicing of the primary transcript or products of proteolytic cleavage of the mature receptor or both (Bole-feysot *et al*., 1998).

1.2.3.5. Prolactin mechanism of action.

Prolactin signal transduction is mediated by a direct signaling system that links the activation of the prolactin receptor at the cell surface to change gene transcription in the nucleus. This pathway is a variant of Jak/Stat (for Janus Kinase/ signal transducer and activator of transcription) pathway which used by other growth factors and cytokines (Watson and Burdon, 1996 and Swaminathan *et al*., 2008).

The extracellular domains of human prolactin receptor isoforms consist of 210 amino acids (Boutin *et al*., 1988 and Boutin *et al*., 1989) which show sequence similarities with other cytokine receptors. The extracellular domains can be further divided into NH2-terminal D1and membrane proximal D2 subdomains (Kelly *et al*., 1991). The most conserved features of the extracellular domain are pairs of disulfide bonds (between Cys12-Cys22 and Cys51- Cys62) in the D1 domain and a "WS" motif (Trp- Ser-x-Trp-Ser) in the D2 domain. The disulfide bonds and the WS motif are essential for the proper folding and trafficking of the receptor , although they are not responsible for binding the ligand itself (Goffin *et al.,* 1988).

The intracellular domains of prolactin receptor are essential for the initiation of the signal transduction mechanisms associated with the prolactin receptor. The isoforms of intracellular domains of prolactin receptor differ in the length and composition but show little sequence similarities with other cytokine receptors. There are two relatively conserved regions termed box1 and box2 (Murakami *et al*., 1991). Box1 is a membrane proximal proline rich motif necessary for the

consensus folding of the molecule recognized by the transducing molecules. Box2 is less conserved and is missing in the short isoform of the prolactin receptor (Goffin *et al*., 1988 and Kelly *et al*., 1991).

Activation of prolactin receptor involves ligand induced sequential receptor dimerization (Figure1.2). Each prolactin molecule contains two binding sites (site 1 involves helices 1 and 4,while site 2 encompasses helices 1 and 3). First, prolactin' s binding site 1 interacts with a prolactin receptor molecule (Goffin *et al*., 1996). The formation of this initial hormone- receptor complex is the prerequisite for the interaction of binding site 2 on the same prolactin molecule with a second prolactin receptor (Bole-feysot *et al*., 1998; Goffin *et al*., 1996).

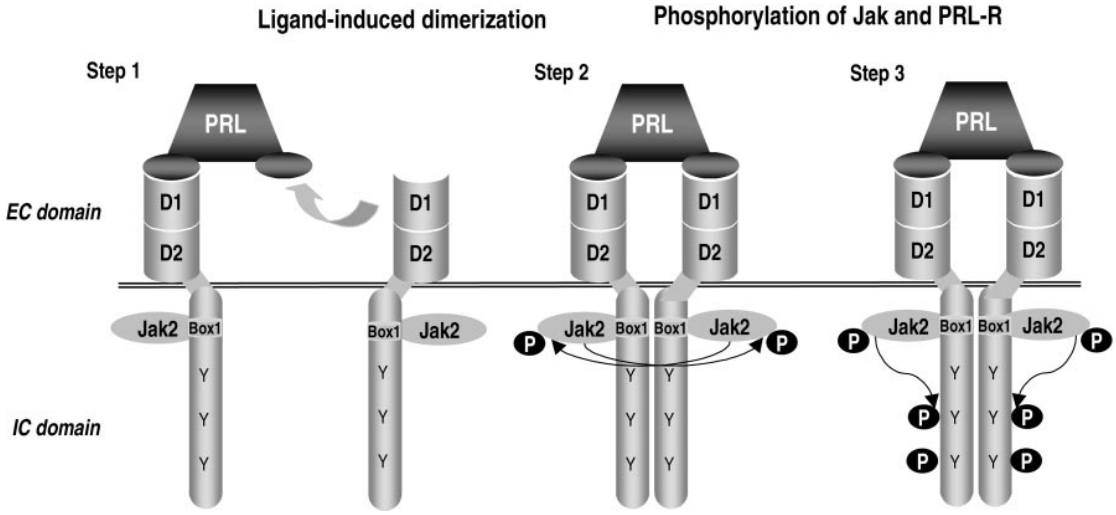


Figure (1.2). Mechanism of prolactin receptor activation. (Freeman, 2000).

The activation of prolactin receptor results in tyrosine phosphorylation of numerous cellular proteins including the receptor itself (Kelly *et al*., 1991; Nagano and Kelly, 1992) although the intracellular domain of the prolactin receptor is devoid of any intrinsic enzymatic activity, the membrane proximal region of the intracellular domain is constitutively associated with a tyrosine kinase

termed Janus Kinas 2 (Jak2) (Campbell *et al*., 1994; Ihle *et al*., 1994 and Lebrun *et al*., 1994). Phosphorylation of Jak2 occurs within 1 min after prolactin binding suggesting a major upstream role for Jak2 (Lebrun *et al*., 1995). There are two major prerequisites for Jak2 activation, the presence of the proline rich box1 motif in the intracellular domain of the prolactin receptor and the homodimeric stoichiometry of the ligand induced prolactin receptor dimers (Ferrag *et al*., 1997; Ghang *et al*., 1998 and Ferrag *et al*., 1998). Activation of Jak2 occurs by transphosphorylation upon receptor dimerization which brings two Jak2 molecules close to each other (Ferrag *et al*., 1998). Phosphotyrosines are of interest since they are potential binding /docking sites for transducer molecules containing SH2.

Jak2 phosphorylates tyrosine residues in different target proteins, the best identified are the receptor itself and a family of transcription factors termed signal transducers and activators of transcription (Stats). These factors are the central transducer molecules of the signal transduction pathways initiated by prolactin receptor activation (Goffin *et al.*, 1998 and Jabbour *et al*., 1998). Stat contains a DNA binding domain, an SH3-like domain, an SH2 like domain, and an NH2- and COOH-terminal transactivating domain (Evans *et al*., 1992). Stat proteins exist within the cytoplasm in a latent or inactive state, they are recruited by cytokine receptor complexes through an interaction involving a phosphotyrosine (on the cytokine receptor and /or the associated Jak) and the SH2 of the Stat protein.

The Stat family currently consists of eight members, three members of Stat family which are Stat1, Stat3 and mainly Stat5 have been identified as transducer molecules of the prolactin receptor. Stat5 was originally identified as mammary gland factor (MGF) and is the major

Stat activated by prolactin receptor. Stat5 has two isoforms, Stat5a and Stat5b, encoded by two different genes with 95% sequence homology and differences only in the COOH-terminal domain. Both isoforms possess a Tyr-694, which is phosphorylated by Jak2 (Gouillex *et al*., 1994). In addition to Tyr phosphorylation, activation of Stat involves serine / threonine phosphorylation as well. The major difference between Stat5a and Stat5b isoforms lies in their serine/ threonine phosphorylation sites (Beadling *et al.*, 1996).

According to the consensus model of Stat activation (Bole-feysot *et al*., 1998; Findori and Kelly, 1995) a phosphorylated tyrosine residue of the activated long prolactin receptor isoform interacts with the SH2 domain of stat (Figure 1.3). Stat, while docked at the receptor, it is phosphorylated by the receptor associated Jak kinase. Then, phosphorylated Stat dissociates from the receptor and hetero or homodimerizes through its phosphotyrosine residues with the SH2 domain of another phosphorylated stat molecule (Figure 1.3). Finally, the Stat dimer translocates to the nucleus and activates a Stat DNA-binding motif in the promoter of a target gene (Carter-su and Smith,1998). The consensus DNA motif recognized by Stat1, Stat3, and Stat5 homo or heterodimers is termed GAS (gama_ interferon activated sequence) which consists of a palindromic consensus sequence: TTCxxxGAA. Once bound, Stat engages several elements of the transcriptional machinery, stimulating gene expression.

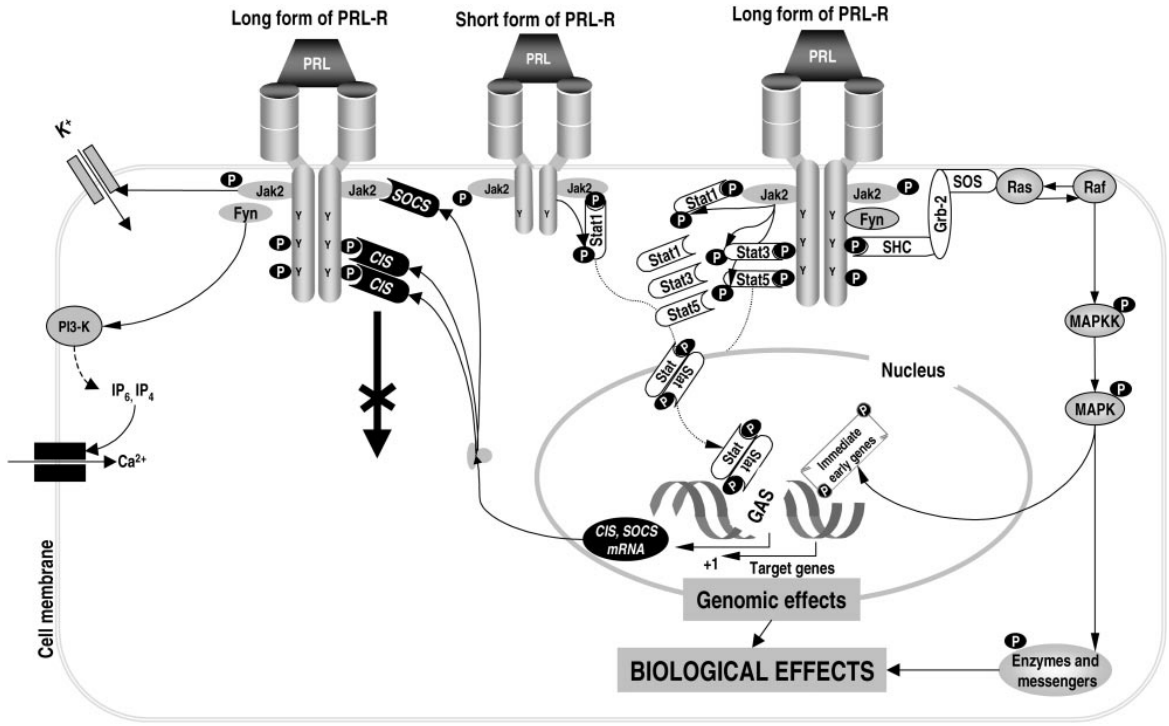


Figure (1.3). Signal Transduction Pathway. (Freeman, 2000).

Although the Jak-Stat cascade is the major signaling pathway used by the prolactin receptor, other transducing pathways are also involved in signal transduction by this receptor. Activation of the prolactin-R also activates the mitogen activated protein kinase (MAPK) cascade, which is involved in the activation of wide range of transcription factors immediate early genes by phosphorylation. Phosphotyrosine residues of the activated long prolactin-R isoform serve as docking sites for adaptor proteins (Shc/Grb2/SOS) connecting the receptor to the Ras/Raf/MAPK cascade (Das and Vonderhaar, 1995). The Jak/Stat and MAPK pathways are interconnected despite these pathways were considered as independent or parallel pathways.

Most transducer molecules are activated by tyrosine phosphorylation (Jaks, Stats etc.) since involvement of tyrosine phosphatases to modulate or down regulate the signaling cascade is expected. It has been reported that SH2 containing Tyr phosphatases

SHP-1 and SHP2 play less role in down regulation of prolactin signaling than in growth hormone or other cytokines (Ali *et al*., 1996; Berchtold *et al*., 1998 and Edwards *et al*., 1998).

Jak/Stat pathways can be inhibited by SH2 containing protein families referred to as cytokine inducible SH2 containing protein (CIS) (Liang and Pardee, 1995; Masuhara *et al.*, 1997) and suppressors of cytokine signaling (SOCS) (Endo *et al*., 1997; Hilton *et al*., 1998). Their main mechanism of action in prolactin receptor signaling is that prolactin induces acute and transient expression of SOCS-1 and SOCS-3. They switch off the prolactin receptor mediated signaling by inhibiting the catalytic activity of Jak2 and activation of Stat proteins . The CIS and SOCS-2 seems to restore the cell's sensitivity to prolactin receptor stimulation probably by suppressing SOC-1 s inhibitory effect.

1.2.4. Prolactin and hyperprolactinemia.

Prolactin is a hormone secreted by the pituitary gland which is located at the base of the brain. It is produced in both females and males. The main function of prolactin is the stimulation of breast milk production after childbirth. However, more than 300 different biological activities of prolactin have been recorded, in large part because of the ubiquitous expression of prolactin receptors including reproduction, water/ electrolyte balance, cell growth and proliferation (Bole- Feysot et al., 1998).

Normally, prolactin is present in small amount throughout the bloodstream of non pregnant females and in males, kept under control by another hormone called prolactin inhibiting factor (dopamine) (Fitzgerald, 2008). Secretion of prolactin may increase mildly during coitus, exercise, sleep, nipple stimulation and pregnancy (Jackson and

Safranek, 2005). In other cases, prolactin can become too high because of a disease or the use of certain medications. Mancini *et al*., (2008) defined the upper threshold of normal prolactin as 25 µg/L for females, and 20 µg/L for males. Furthermore, Corona *et al*., (2009) defined hypoprolactinemia as prolactin levels below 3µg/L in females, and 5µg/L in males.

Hyperprolactinemia is a condition in which higher than normal levels of the hormone prolactin circulates in the bloodstream of not pregnant females and in males. It is probably one of the most common endocrine disorders related to pituitary function (Surks *et al*., 2004). Hyperprolactinemia is an extremely common disorder especially among reproductive age females. Its prevalence is especially high in females presenting with reproductive or menstrual dysfunction (Nilsson and Hellberg, 2006).

One of the first signs of hyperprolactinemia in females are irregular menstrual cycles. Another common symptom is galactorrhea which is the occurrence of a milky discharge from the breast in non pregnant females. The discharge is the result of persistent high prolactin levels stimulating the mammary gland for milk production. In some females galactorrhea may occur spontaneously whilst in others it may only occur if they squeeze their nipples (Oner, 2013). In some cases of severe hyperprolactinemia the estrogen level can be decreased to a point where the loss of bone calcium can occur (Melmed *et al.*, 2011).

1.2.5. Causes of hyperprolactinemia.

1.2.5.1. Prolactinoma (pituitary adenoma).

Pituitary adenomas are the most common tumor type in the pituitary gland. In some people, a small group of cells may form

a cyst in the pituitary gland that produces elevated levels of prolactin these cysts are called prolactinoma or pituitary adenomas. This tumor is mostly benign (adenomas) meaning not invasive and not metastatic. It is more common in females than males. The adenomas can be seen and measured using MRI (magnetic resonance imaging) and classified based on their size (Wix-Ramos *et al.*, 2011).

Small adenomas are known as microadenomas they measure less than one centimeter in diameter, this is the most common type of adenoma found. Microadenomas can even be present in healthy people who do not have high prolactin levels and can be treated with medications. They do not grow large and do not need to be treated if hormone levels are normal. Microadenomas usually follow a benign course and rarely progress to macroprolactinomas however, in rare cases microadenoma may transform to other tumors (Schlechte, 2007).

Adenomas larger than one centimeter are called macroadenomas. If untreated it can grow further and start to compress the nearby tissues and structures causing life threatening events or even fatal outcome. The closest structures are the optic nerves, internal carotid arteries. If a macroadenoma causes compression of the optic nerves, partial blindness can result for this reason, It is important to treat macroadenomas whether or not females are interested in getting pregnant. Medication can be used to treat them, but if that fails, surgery may be necessary (Bushe *et al*., 2010).

1.2.5.2. Hypothyroidism.

Hypothyroidism is a condition in which inadequate amount of thyroid hormone is produced. It is the most common medical condition that can cause hyperprolactinemia (Cooper *et al*., 1984; Canaris

et al., 2000; Hollowell *et al.*, 2002 and Bahar *et al.*, 2011). The hyperprolactinemia of hypothyroidism is related to several mechanisms, in response to the hypothyroid state a compensatory increase in the discharge of central hypothalamic thyrotropin releasing hormone (TRH) results in increased stimulation of prolactin secretion. Treatment with thyroid hormone supplements will results in correction of both thyroid feedback and the high prolactin levels (Micinsk *et al*., 2006 and Binita *et al.*, 2009).

1.2.5.3. Macroprolactinemia.

Macroprolactinemia is a large pituitary tumor greater than 10 mm in diameter. Asymptomatic patients with intact gonadal and reproductive function and moderately elevated prolactin levels may have macroprolactinemia (Vallette-Kasic, 2002; Shimatsu and Hattori, 2012). Hypersecretion of prolactin by lactotroph cells of the anterior pituitary cause hyperprolactinemia patients, they may have radiologically undetected microprolactinomas, but some of them may present other causes of hyperprolactinemia characterized as a symptom of macroprolactinemia with a predominance of higher molecular mass prolactin forms (big-big prolactin, MW>150 KD) (wilson *et al*., 2005).

The pathophysiology of macroprolactinemia is based on a mechanisms of the increased antigenicity of these molecules leading to the appearance of autoantibodies against prolactin which can consequently reduce the bioactivity of prolactin and provide extended half-life (Cavaco *et al.*, 1995; Pascoe- lira *et al.*, 2001; De schepper *et al*., 2003 and Hattori *et al*., 2010).

1.2.5.4. Stress.

A high prolactin level can sometimes be related to physical stress even drawing blood can by itself cause someone to produce and

immediate prolactin release. In general prolactin elevation can also detected in response to strong or sudden external stimuli such as stressful environmental conditions or can be related to physchological reasons. Life events such as changes in subject's social or personal environment indicated that these stressful conditions may provoke hyperprolactinemia. Even an exposure during childhood to a stressful environment may be associated with hyperprolactinemia and/or galactorrhea later in life as a response to specific environmental changes (Sonino *et al.*, 2004). Several external stress factors may contribute to the occurrence of hyperprolactinemia. In theory, stress might have been involved in facilitation of a clonal proliferation of a single mutated cell and cause prolactinomas or stress might trigger neuroendocrine changes involving dopamine and/or serotonin, which both can consequently affect prolactin release (Fava, 1981; Freeman *et al.*, 2000 and Verhelst, 2003).

1.2.5.5. Medications.

Some medications can cause higher levels of prolactin to be produced. The most common medications that do this are known as anti-psychotic medications. Other medications which may increase prolactin levels include:

- Some types of anti-depressant (amoxapine, imipramine).
- Some types of sedatives.
- Dopamine receptor antagonists (metoclopramide, phenothiazines).
- Estrogen.
- Oral contraceptives.
- Some types of blood pressure medications (methyldopa, reserpine).
- Medication for nausea (reglan, metoclopramide).

(Nussey and Whitehead, 2001).

1.2.6. Hyperprolactinemia and infertility.

Infertility is defined as the inability to conceive after one year of unprotected intercourse. It has two types, primary infertility; is a term used for couple who have never achieved a pregnancy. Secondary infertility referred to couple who have previously succeeded in achieving at least one pregnancy even if this ended in abortion. The major causes of infertility include ovulatory dysfunction, tubal and peritoneal pathology, male factor and uterine pathology (Mohan and Sultana, 2010).

Hyperprolactinemia is a common problem in reproductive dysfunction affecting about one-third of infertile females (Corenblum, 1993). Hyperprolactinemia leads to hypogonadism, which in turn interfering with the action of the gonadotrophin (FSH and LH) at the ovarian level, then impaired gonadal steroid secretion which alters positive feedback effects on the hypothalamic and pituitary levels. This leads to lack of gonadotrophin cyclicity (FSH and LH decline) and to infertility (Thorner and Besser, 2008; Mohan and Sultana, 2010). Another cause of infertility seen during hyperprolactinemia is the low estradiol production, Prolactin elevation can inhibit follicular estradiol production and this results in infertility (Uilenbroek and Linden, 1984; Kalsum and Jalali, 2002; Jameel, 2010).

1.2.7. Treatment of hyperprolactinemia.

Prolactin levels can often be corrected by stopping suspected medication or switching to a different medication type. Correction of hypothyroidism is also effective and specific to reduce prolactin levels. If prolactin levels are persistently high, they can be effectively

treated with a group of medications known as dopamine agonists (Melmed, 2011).

1.2.7.1. Dopamine agonists drugs.

1.2.7.1.1. Bromocriptine (Parlodel).

The first dopamine agonist drug used was bromocriptine. It is an effective and inexpensive medication for high prolactin levels, it has a similar mode of action to dopamine in stimulating dopamine receptors on the prolactin secreting pituitary cells- D2 receptors. Stimulation of these receptors leads to inhibition of both secretion and synthesis of prolactin. Parlodel is usually taken at bedtime with a snack this is because parlodel will occasionally cause dizziness or stomach upset, so taking it before sleep and with food will reduce these side effects. Generally with time the side effects, stop anyway (Oner, 2013).

1.2.7.1.2. Gabergoline (Dostinex).

Gabergoline is more effective than bromocriptine in normalization of prolactin levels and resuming normal ovulatory cycles (Dos Santos Nunes *et al*,. 2011). Gabergoline is more expensive than bromocriptine so it is usually used when bromocriptine is ineffective or female cannot tolerate the side effects. Gabergoline is a longer acting medication it is usually given twice a week instead of every day. Dopamine agonist medications are useful for reducing prolactin levels tumor size and to restore gonadal function in patients with prolactinoma (Kreutzer *et al*., 2008). Patients who cannot tolerate high doses of drugs or who are unresponsive to dopamine agonist therapy should offered trans sphenoidal surgery. Radiation therapy is useful for patients in whom surgical treatment fails or for those with aggressive or malignant prolactinomas (Wang *et al.,* 2012).

3. Results and Discussion.

3.1.The effect of hyperprolactinemia on fertility.

Hyperprolactinemia is a major part of infertility problems that consumed a great effort and expensices in the treatment of couples. Many reasons may involve in such physiological disorder, non of them was considered as main one but some of them appeared frequently to affect fertility in females and seem to play a major role in elevation of prolactin. Screening of 150 females with hyperprolactinemia according to their age and the types of fertility hormones gave the results shown in table (3.1).

Table (3.1): Measurement of prolactin, LH and FSH in females with hyperprolactinemia.

No.	Hormone	Hormone normal concentration	Measured concentration	Deviation in hormone concentration
1	Prolactin	2 – 10 mU/ml	40 – 100 mU/ ml	38 – 90
2	LH	3 – 6 mU/ml	0.2 - 1.5 mU/ ml	2.8 - 4.5
3	FSH	2 – 8 mU/ml	0.1 – 1 mU/ ml	1.9 – 7

As shown in table (3.1) measurement of three highly involved fertility hormones revealed abnormal change in their ratio. LH and FSH were found to decrease in test subjects when compared with normal while prolactin showed high elevation to about six folds in test subjects with

hyperprolactinemia infertility problem. This was statistically analyzed as shown in table (3.2).

Table (3.2): Statistical analysis of fertility hormones measurement in females with hyperprolactinemia categorized according to age.

	(Mean±SD)		
Groups / Hormones	LH	FSH	Prolactin
20-30	A 0.591±0.347	A 0.563±0.269	A 62.470±18.758
31-40	A 0.632±0.269	A 0.618±0.256	A 68.365±17.476
41-50	A 0.660±0.240	B 1.177±3.385	A 66.050±18.258
Control	B 4.400±1.020	C 4.680±1.708	B 3.206±1.429

Differences letters A,B,C are significant at (P<0.05) to comparison columns.

A graphical representation is shown in figures (3.1), (3.2) and (3.3) regarding LH, FSH and prolactin respectively.

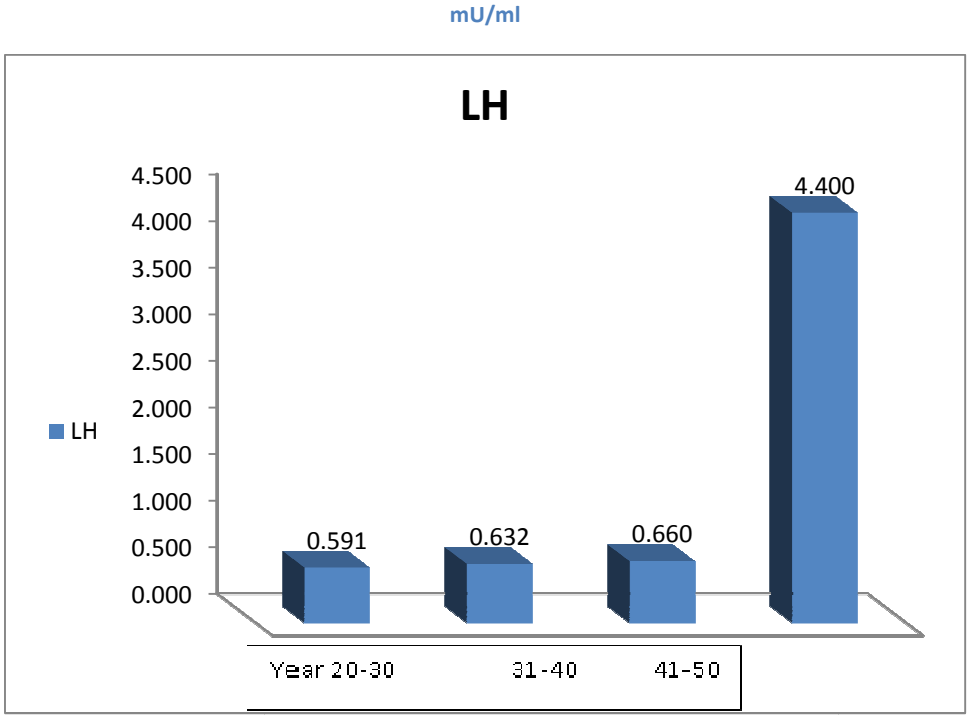


Figure (3.1): LH levels in infertile and control groups categorized according to age.

The figure shows that LH level was decreased in each age group of test subjects compared to control.

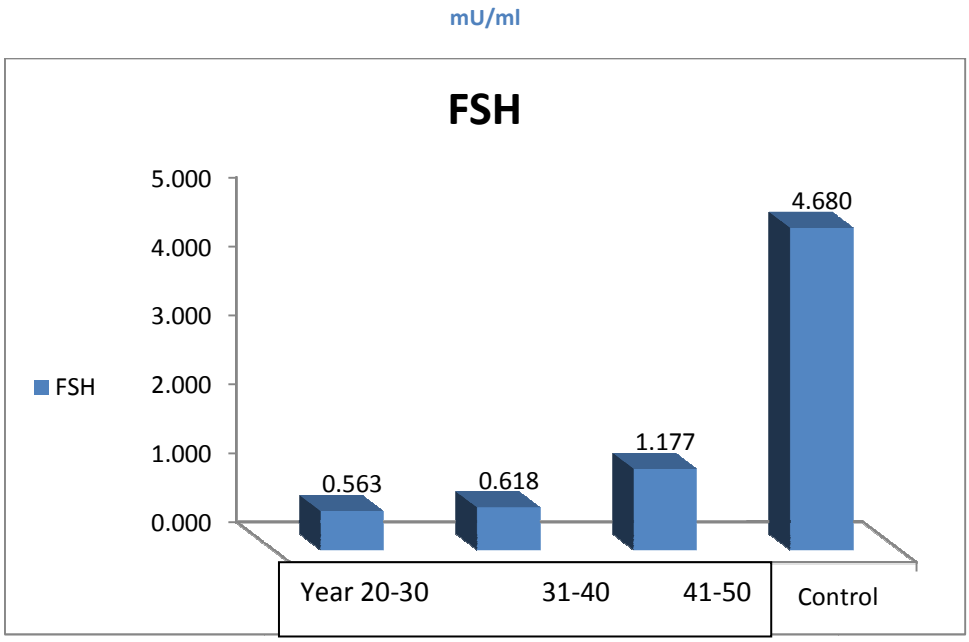


Figure (3.2): FSH levels in infertile and control groups categorized according to age.

The figure shows that FSH level was decreased in each age group of test subjects compared to control.

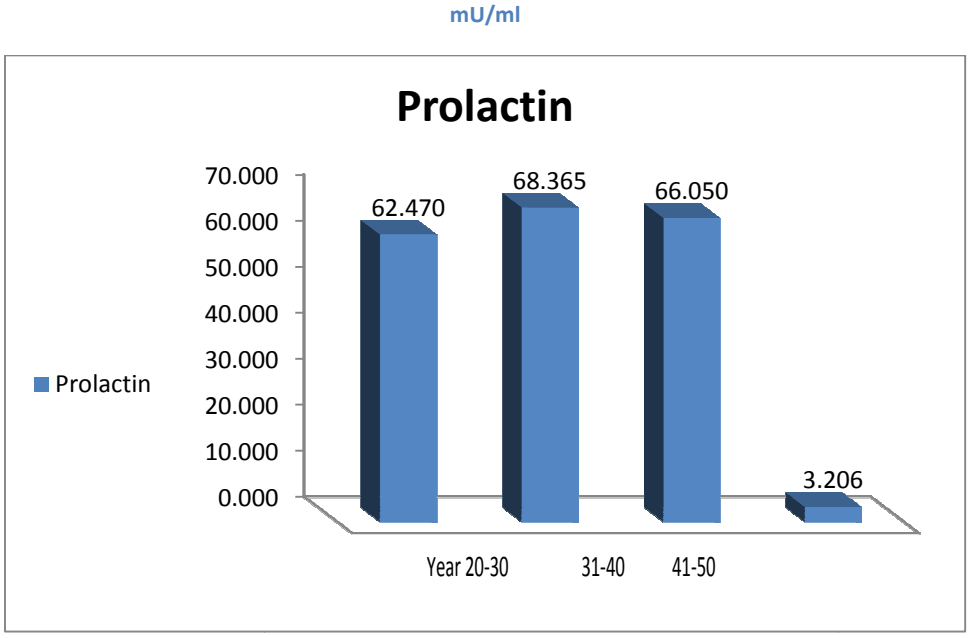


Figure (3.3): prolactin levels in infertile and control group categorized according to age.

Regarding prolactin comparison among age groups the results showed an obvious elevation within age group (31-40) years old than

those (20-30) and (41-50) years old. Such observation may be explained on the basis of maturation of sexual system and stability of hormone release and response of the body, once there is an abnormal elevation it will stabilize at this age since females are at the optimal reproduction age, so such abnormalities will be obvious and pose a problem for a long time. This observation and the elevated level are less in age 20-30 years with standard deviation (SD) of 5.8 compared with age group 30-40 years old so female objects are at the end of teenage period and heading to life span of stability in reproductive system hormones. Noticing that age group 40-50 years old the prolactin level is with SD of 2.3 only when compared with 30-40 years old group. This supports the former explanation regarding 30-40 years old group, once such abnormal elevation started it will continue even with age progress and many remain stable till 50 years old age it was treated, or it may be related to other disease in the immune system, or arthritis.

An explanation for such phenomenon may be built on several reasons: first, one may be attributed to an imbalance of the endocrine system related to prolactin releasing factor that seems to be with high level (Kalsum and Jalali, 2002), second, it seems to be a problem with feedback inhibition system that should control hormone level within the body once there is an elevation or decrease (Nussey and Whitehead, 2001). Third, some researchers attributed such abnormality to prolactin receptors at cell membrane which disrupt normal response to hormone level in the blood causing a crash at its level control. Fourth, some of female subjects studied were candidate to show breast cancer depending on their family history and clinical examination which may explain control disruption of hormone level. Regarding feedback inhibition or prolactin receptor response due to

transformation phase of mammary cell from normal cell to malignant one as preliminary histopathology examination showed in some patients (Arendt *et al*., 2011).

According to Thorner and Besser, (2008) it has been suggested that hypogonadism seen in hyperprolactinimic females is due to the high circulating levels of prolactin interfering with the action of the gonadotrophins at the ovarian level and impairing normal gonadal steroid secretion which in turn alters the positive feedback effects at the hypothalamic and pituitary levels. This leads to the lack of gonadotrophin cyclicity and to infertility. A similar association between increased prolactin levels and reduction in LH and FSH occurs during infertility in females in pathological hyperprolactinemia (Neilly, 1987).

This study coincides with the study of Givens *et al*., (2006) that showed females with higher levels of prolactin and luteal phase defects have lower levels of FSH and LH during their menstrual cycle. Bevan *et al*.,(1992) suggested that a decline in gonadotrophin in hyperprolactinimic females compared to normoprolactinimic females indicates that an association between gonadotrophin deficiency and hyperprolactinemia and this may be an indirect sign of functional hypothalamic pituitary interruption due to the inhibitory effect of prolactin on gonadotrophin release.

In addition, this study agrees with the study of Tjeerdsma *et al*., (1996) which reported a decrease in serum LH, FSH and Estradiol (E2) levels in hyperprolactinimic females compared to normoprolactinimic females, and the study of Kalsum and Jalali, (2002) that showed highly significant increase in serum prolactin level with a significant decrease in serum LH and FSH levels was observed in hyperprolactinimic females in

different menstrual phases. According to Mohammed, A. (2003) increased prolactin levels may be the cause of low estrogen and progesterone concentrations that resulted in a decrease in serum LH and FSH in infertile females. According to Emo Kapae, M. (2005) there may be a failure at the hypothalamus or pituitary (hypogonadotropic-hypogonadism) that leads to decreased in serum FSH and LH with increased in serum prolactin levels and resulted in infertility. Moreover, this study coincides with study of Mohan and Sultana, (2010) who suggested that a decrease in the level of LH in the midcycle clearly indicates that there is a possibility of anovulation which results in infertility. The elevated prolactin values in infertile females clearly indicate that there is a mechanism operating at the anterior pituitary level which shows an abnormal distribution of FSH and LH that may further explain the abnormal or delay ovum maturation.

3.2. Molecular analysis of hyperprolactinemia.

Many abnormalities in the endocrine system and hormones might be based on genetic abnormality at the molecular level, which made this study to concentrate on main element that was not referred to by researchers, to estimate the severity of the disease by the detection and isolation of prolactin related RNA from serum other than blood (Yue *et al.*, 2010). It was possible to isolate RNA from serum with a concentration of 300 nanogram (ng) and purity of 1.5–2 which was used for complementary DNA (cDNA) specific amplification. The presence of high levels of prolactin related RNA in serum may pose the seriousness of the disease and an indicator to the presence of a fault at the molecular level since such isolation of RNA was recommended from whole blood by some researchers.

3.2.1. Evaluation of PCR method for prolactin related RNA amplification.

Two methods were used for cDNA specific amplification, the conventional method and hot start procedure. By comparing both methods, the hot start method was better in yield and amplification since the product concentration was about 100 mg a with wide area of occupation and sharpness when electrophoresed on agarose gel as shown in figure(3.4) and figure (3.5).

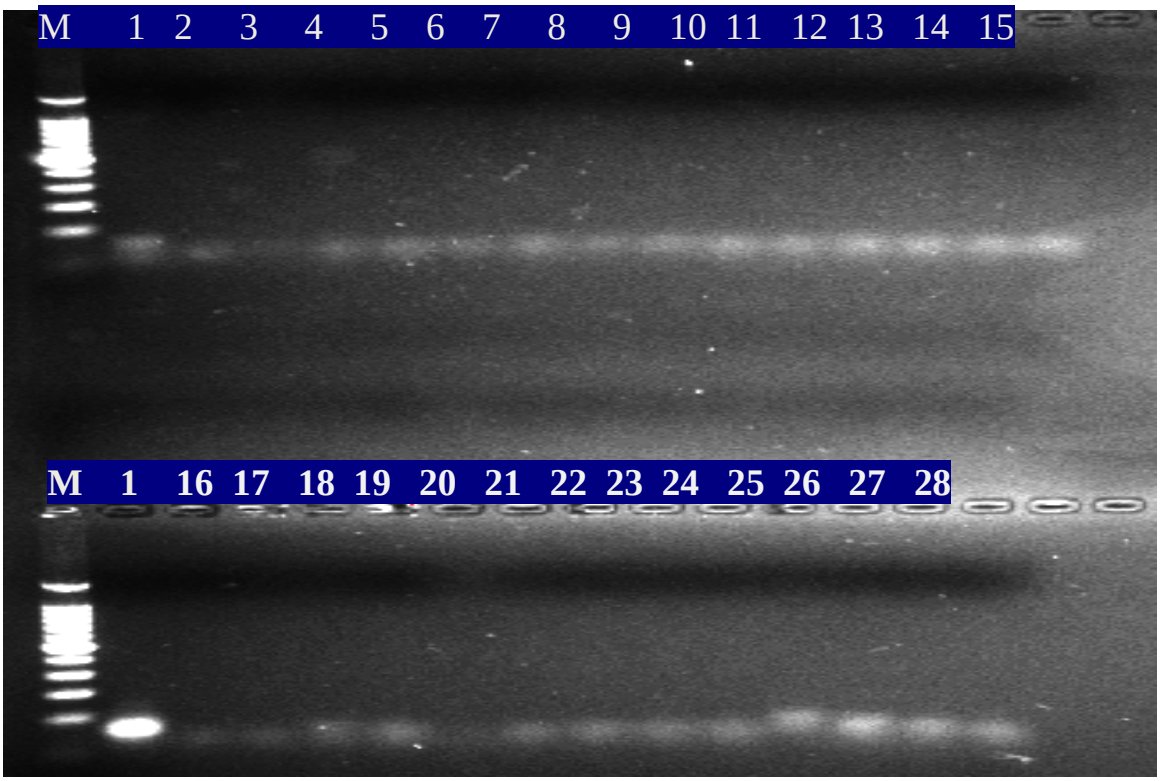


Figure (3.4): Agarose gel electrophoresis (2% agarose, 90 minutes, 90 V) for prolactin PCR products visualized under U.V light after staining with ethidium bromide. M: 100 bp DNA marker; lane 1: for control and lanes: 2-28 for hyperprolactinimic infertile females.

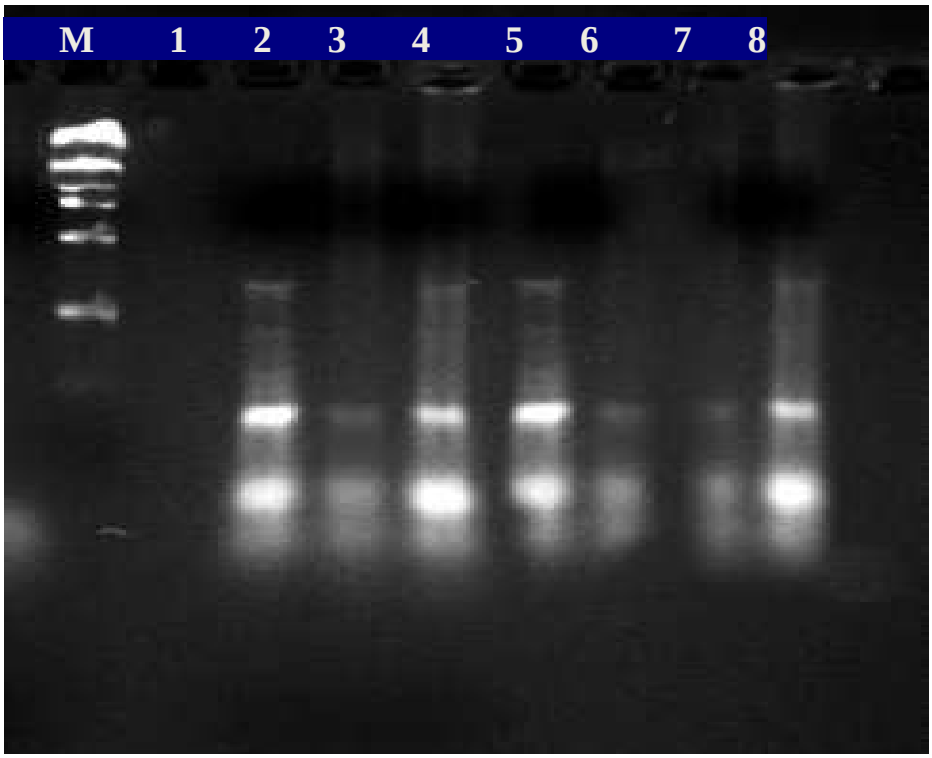


Figure (3.5): Agarose gel electrophoresis for prolactin PCR products visualized under U.V light after staining with ethidium bromide. M: 100 bp DNA marker; lane 3and7 for control and lanes 2, 4, 5, 6, and 8 for hyperprolactinimic infertile females.

There is a rational explanation of using the hot start method in PCR amplification such explanation may be attributed to RNA conformation in hyperprolactinimic patients. High level secretion and proposed complication in RNA shape may require such method by PCR resolve such complication and obtain reliable results (Caelers *et al*., 2004).

3.2.2. Evaluation of primers efficiency and specificity.

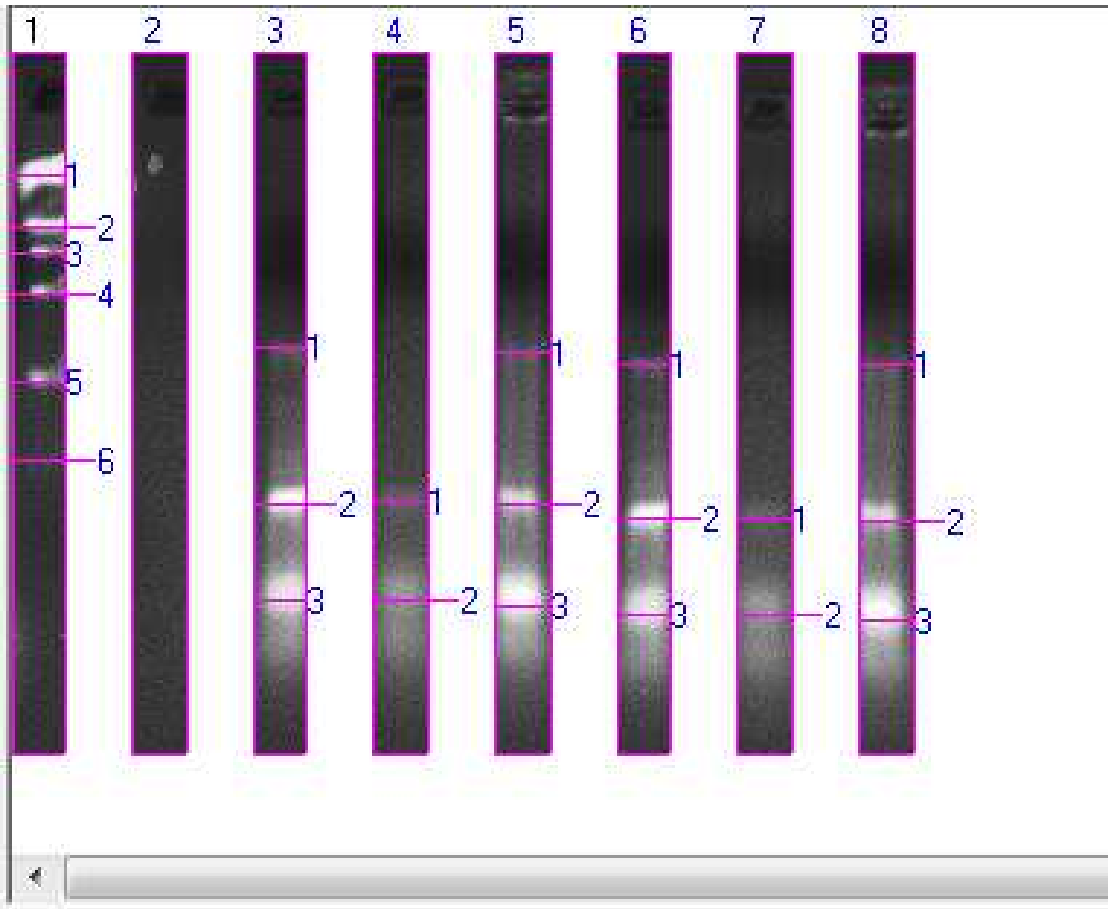
Primers used in this study were previously designed to amplify the prolactin gene. Two of these primers gave a distinct result and product by PCR amplification, those designated as hpRL1and hpRL2 while TBP did not give any results by PCR amplification. Previously, both first primers were designed to amplify specific exon region at prolactin gene

by Hattori *et al*.,(2006) while TBP was designed to detect the expression domain at prolactin gene by Reuwer *et al*., (2011). Amplification of sites for both first two primers may indicated insignificant in the change percentage of prolactin gene to which they related, while the lack of product that regarding the third primer may illustrate the dramatic change in the expression level site of the gene.

3.2.3. Interpretation of PCR results using prolactin specific primers.

Specific PCR amplifications were performed using the conventional PCR method and hot start method. All samples of the study were subjected to this process using the three pre-designed primers. The amplified samples were shown in figures (3.6) and (3.7) were the control (normal), patients with high prolactin levels (Group 30-40 years old) and the other two groups (20-30 years old group and 40-50

years old group).

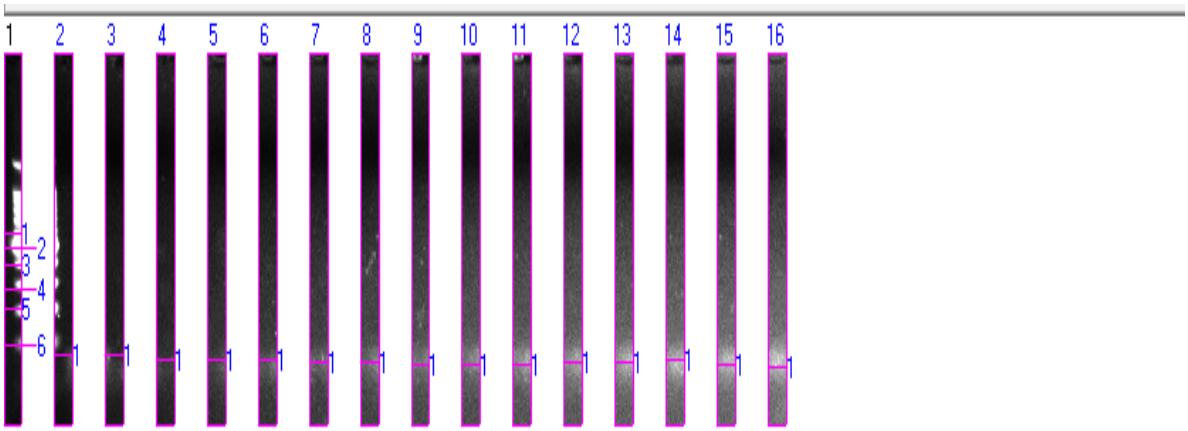


	M.W.						
	L1	L3	L4	L5	L6	L7	L8
1	1.000	0.631	0.448	0.626	0.616	0.426	0.616
2	0.900	0.444	0.322	0.444	0.426	0.304	0.422
3	0.800	0.322		0.315	0.304		0.296
4	0.700						
5	0.600						
6	0.500						

Figure (3.6): Analysis of DNA fragments resulted from RT-PCR of RNA from subjects under study.

The figure shows the position and size of DNA band in kbp photographed from electrophoresis. Analysis was performed using PhotoCapt DNA analysis software.

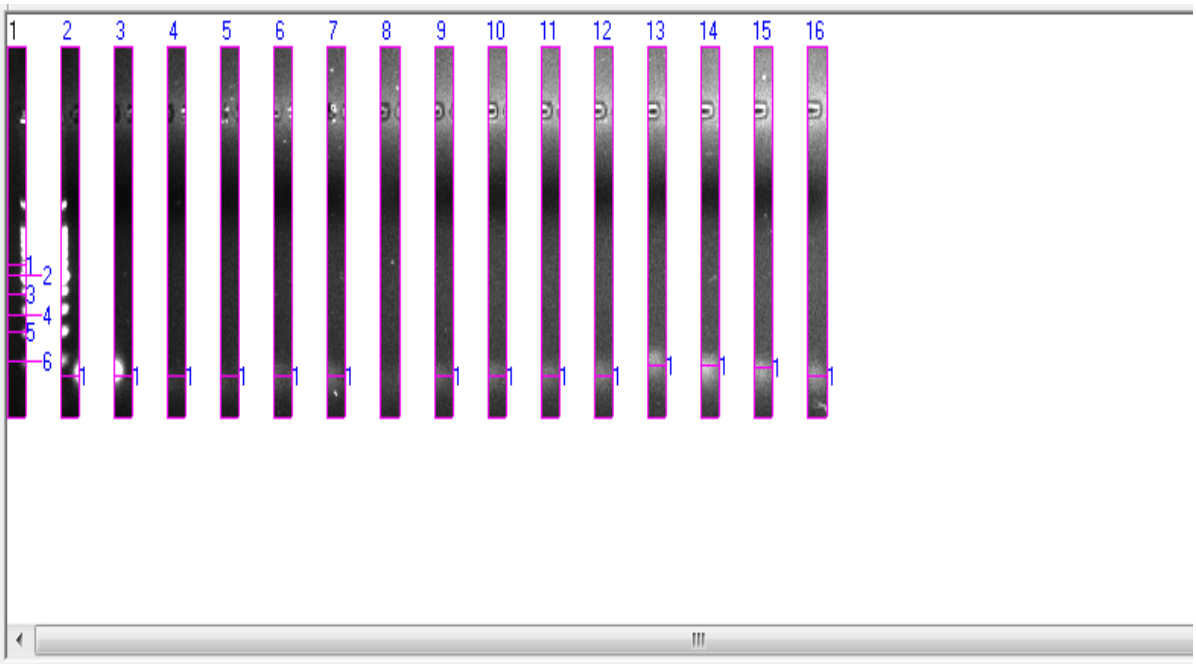
The obtained data in figures (3-5) and (3-6) are explained through comparing the bands of figure (3-6) (lines 8) with the normal (lanes 3 and 7), exon 3 was highly expressed and attributed to the involvement of prolactin as a factor of B-lymphoblastoid maturation (Biga *et al*., 2005).



	M.W.															
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16
1	0.600	0.073	0.073	0.060	0.060	0.060	0.053	0.053	0.047	0.047	0.047	0.053	0.053	0.060	0.047	0.040
2	0.500															
3	0.400															
4	0.300															
5	0.200															
6	0.100															

Figure (3.7): Analysis of DNA fragments resulted from RT-PCR of RNA from subjects under study.

The figure shows the position and size of DNA band in kbp photographed from electrophoresis. Analysis was performed using PhotoCapt DNA analysis software.



	M.W.														
	L1	L2	L3	L4	L5	L6	L7	L9	L10	L11	L12	L13	L14	L15	L16
1	0.600	0.050	0.050	0.050	0.050	0.050	0.050	0.050	0.050	0.050	0.050	0.086	0.086	0.079	0.050
2	0.500														
3	0.400														
4	0.300														
5	0.200														
6	0.100														

Figure (3.8): Analysis of DNA fragments resulted from RT-PCR of RNA from subjects under study.

In figure (3.7) the primer used to amplify specific region at which mRNA of coding region should appear. In normal (control at lane 2) the band appeared to be with high yield of product, sharp edges and distinct among others while in patients (lane 3-16) the band appeared with less luminance and low yield. Even among patients lanes 3, 4, 5 to which DNA samples are from patients at age group (31 - 40 years old) with the most high level of prolactin, it appeared to be more fante compared with age group (41 – 50 years old) lane (5, 6, 8, 9) while patients with age group (20 - 30) lane (12 - 15) showed more

yield of PCR product and appeared to be more distinct among patients samples from previous categories.

It is not worthy regarding hpRL3 primer which gave no product by PCR, and was originally designed for prolactin receptor abnormality (Reuwer *et al.,* 2011). This may indicate the presence of normal prolactin receptor system among patients in the study.

From all above data, it was found that primers with positive results were able to amplify a part of whole gene that were designed for cDNA synthesized during experiments. The size resulted from this amplification was not suitable for sequences, thus restriction analysis is recommended for further analysis.

3.3. Protein profile Analysis of hyperprolactinemia

With the approach of the post genomic area and popularity of recombinant DNA technology, there has been resurgence in the use of gel electrophoresis to identify and characterize various gene products. Electrophoresis is relatively simple, rapid, and highly sensitive tool to study the properties of proteins thus, proteins of serum from hyperprolactinemic patients were analyzed using cellulose acetate paper. This method is simple and generally satisfactory for distinguishing the common types of protein and it is mostly used in laboratories for rapid detection of serum disorders.

Serum samples from patients and their relatives were taken proteins were separated and subjected to electrophoresis by cellulose - acetate paper to identify abnormal levels among hyperprolactinemic patients and investigate the reason behind such disorder. The results obtained are shown in figure (3. 9).

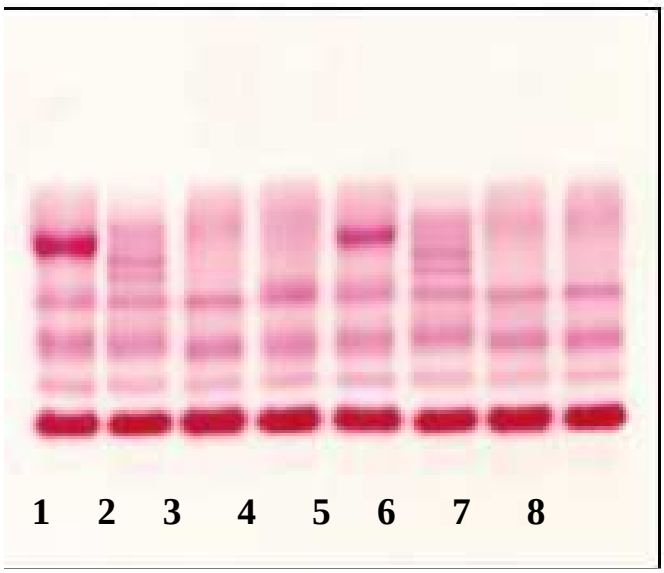


Figure (3.9): protein electrophoresis, lanes 2 and 6: normal (control), lanes 1, 3,4,5,7 and 8: patients with hyperprolactinemia.

Result obtained showed that prolactin elevation caused an increase in albumin level, this protein knows to run function as a carrier of hormones (Zippelius *et al*., 1997). Such albumin elevation is a normal response of body toward hyperprolactimia to be able to cope with such case of abnormal elevation of prolactin.

2. Materials and Methods.

2.1. Materials.

2.1.1. Apparatus.

The apparatus used in the study are shown below

Apparatus	**Company / country**
Analytical balance	FC-400 / China
Autoclave	Tomy / Japan
Bench centrifuge	Uni-Media / Korea
Electrophoresis equipment	Consort / Belgium
Gel –documentation	Bio-Red / U.S.A
Heater – magnetic stirrer	Stuart / England
Incubator	Sanyo / Japan
Microcentrifuge	Hettich / Germany
Microwave	LG / Korea
Minividas	Bio mearex / France
Nanodrop spectrophotometer	Techne / U.K.
Oven	Sanyo / Japan
PCR master cycler gradient	Techne / U.K.
pH meter	Martini / Japan
Sensitive balance	Mettlev / Switzerland
U.V. transilliuminator and Camera	Flowgen / U.K.
Vortex	Scientific industries / U.S.A.
Water bath	Memmert / Germany

2.1.2. Chemicals.

Promega / U.S.A		
DNA Ladder	Green Master Mix	Proteinase K
Merck / Germany		
Formamide		
Glycine	Methanol	
Sigma / U.S.A.		
Diethyl pyrocarbonate (DEPC)	EDTA	Ethidium bromide
3-(N- morpholino) propan sulforic acid (MOPS)	Tris – HCL	
Fluka / Switzerland		
Boric acid	Bromocresol purple	
Acetic acid	Chloroform	Formaldehyde
2- Mercaptoethanol	Sodium hydroxide	Tris – base
Trichloroacetic acid	Barbitone (diethylbarbituric acid)	Sodium diethyl barbiturate
Methanol	Glacial acetic acid	
BDH / England		
Bromophenol blue	Ethanol	Glycerol
Polyethylene glycol	Ponceau S	Sodium acetate

2.1.3. Kits.

Kits	Company	Country	Cat- NO.
RNA Purification mini kit	Favorgen	Taiwan	001-1
Reverse transcription system kit	Promega	U.S.A	A5001
LH	Bio - Merieux	France	06267K
FSH	Bio - Merieux	France	06268I
Prolactin	Bio - Merieux	France	06269H

2.1.4. Collection of samples.

Blood samples were collected from 150 hyperprolactinimic infertile women and 50 healthy women as a control. Their ages ranged between (20-50) years. A volume of a 5ml of peripheral blood was collected by vein puncture into sterile tube and incubated for 30 min. to allow blood clotting. The serum was separated from blood cells by centrifugation at 2000 rpm for 10 min. at room temperature, then the serum was stored at -20c until used. These samples were obtained from infertility center of Al-Yarmok Teaching Hospital, St. Rafael Hospital and Al-Kindey Teaching Hospital starting from November 2012 to February 2013.

2.1.5.Solutions.

2.1.5.1. Sterile DEPC- treated solution.

A volume of 0.1 % DEPC was added to water, mixed overnight, and then autoclaved for 20 min to destroy DEPC by hydrolysis.

2.1.5.2. Hormones kit

2.1.5.2.1. Fertility hormones (Butt and Blunt, 1988)

The following kits were used for fertility hormones.

2.1.5.2.1.1.FSH

FSH levels were measured by the Bio – Merieux kit Sa. 06268I marcy I' Etoile – France, as instructed by the manufacturer.

2.1.5.2.1.2. LH

LH levels were measured by the Bio – Merieux kit Sa. 06267K marcy I' Etoile – France , as instructed by the manufacturer.

2.1.5.2.1.3. Prolactin

prolactin levels were measured by the Bio – Merieux kit Sa. 06269H marcy I' Etoile – France , as instructed by the manufacturer.

2.1.5.3. RNA Purification Mini kit.

Favor Prep™ Total RNA Purification Mini Kit from Favorgen, Taiwan (001-1), ready to use that contained the following components:

- FARB Buffer (45ml).
- Wash Buffer1(60ml).
- Wash Buffer2 (35ml).
- Rnase- free ddH2O (6ml).

2.1.5.4. Determination of RNA yield and purity (Sambrook and Russel , 2001).

2.1.5.4.1. Gel electrophoresis analysis of RNA.

The following buffers and solutions were used for gel electrophoresis analysis of RNA.

- **10X MOPS electrophoresis buffer.**

MOPS pH 7.0	0.2 M
Sodium acetate	20 Mm
EDTA pH 8.0	10 Mm

Three solutions were prepared separately for the MOPS electrophoresis buffer. First solution was prepared by dissolving 41.8 gm of MOPS in 700ml DEPC – treated water. The pH was adjusted to 7.0 by addition 2N of NaOH. A second solution was prepared from 1M sodium acetate.A third solution was composed of 0.5M EDTA (pH 8.0). The final buffer was made by adding 20ml from the second solution and 20ml of the third solution to the first one, and the volume was completed to 1000ml. The final buffer was sterilized by filtration through a 0.22µm Millipore filter then kept at room temperature.

- **RNA sample buffer.**

  The RNA sample buffer was prepared by mixing 10 ml formamide , 3.5 ml (37%) formaldehyde and 2 ml (10X) MOPS buffer . This buffer was stored at – 20 ^{o}C.

- **The RNA loading buffer.**

RNA loading buffer was prepared by mixing 50% glycerol , 1mM EDTA and 0.4% Bromophenol blue.

❖ **RNA Marker.**

RNA Marker range 0.28 – 6.6 Kb, Ready – to – use, it was used for agarose (formaldehyde) gel electrophoresis.

2.1.5.5. Reverse Transcription System Kit.

GoScript™ Reverse Transcription system kit from Promega, U.S.A ready to use. It wascontained the following components:

- GoScript™ Reverse Transcriptase
- GoScript™ 5x Reaction Buffer
- $MgCl_2$
- Recombinant RNasin Ribonuclease Inhibitor
- PCR Nucleotide Mix
- Random Primers
- Nuclease- Free Water

2.1.5.6. Green Master Mix.

Go Taq® Green Master Mix is a premixed ready to use solution containing bacterially derived Taq DNA polymerase, dNTPs, $Mgcl_2$ and reaction buffers at optimal concentration for effective amplification of DNA templates by PCR.

2.1.5.7. Go Taq® Hot Start Green Master Mix.

Go Taq® hot start green master mix is a premixed ready to use solution containing Go Taq® hot start polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

2.1.5.8. Human prolactin primers.

Sequences of the primers used to amplify aportion of the PRL gene.

Oligonucleotides	Sequence (5'-3')	Tm	GC%	Reference
Forward primer	AAC ATG AAC ATC AAA GGA TCG	51.0	38.0%	(Hattori, 2006)
Reverse primer	TTA GCA GTT GTT GTT GTG GAT	52.5	38.0%	
Forward primer	GGG TTC ATT ACC AAG GCC ATC	56.0	52.3%	(Sabharwal *et al.*, 1992)
Reverse primer	CAG TCG GTC CAA GTA GGA CCT	58.2	57.1%	
Forward primer	GTT CCA GCG CAA GGG TTT CTG GT	62.2	56.5%	(Reuwer, 2011)
Reverse primer	GAG TCA TGG CAC CCT GAG GGG AG	63.4	65.2%	

.2.1.5.9. DNA gel electrophoresis (Maniatis *et al.*, 1982).

The following solutions were used for DNA electrophoresis.

2.1.5.9.1. (0.5 M) EDTA (pH 8.0).

A weight of 2.922 gm EDTA was dissolved in 15 ml deionized distilled water, and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 using (10N) NaOH, then the volume was completed to 20 ml using deionized distilled water then sterilized by autoclaving.

2.1.5.9.2. (5X) TBE buffer.

Tris – base	54 gm
Boric acid	27.5 gm
EDTA (0.5M) pH 8.0	20 ml
deionized distilled water	1000 ml

Then was sterilized by autoclaving

2.1.5.9.3. Loading buffer.

Tris – base pH 8.0	0.025 M
Glycerol	50%.
Bromocresol purple	0.25%

2.1.5.9.4. Ethidium bromide stock solution.

Ten milligrams of ethidium bromide dye were dissolved in 1 ml of deionized distilled water stirrer on magnetic stirrer for several hours until the dye has dissolved. The container wrapped with aluminum foil or kept in a dark bottle and stored at $4^{o}C$

2.1.5.9.5. DNA ladder (100 bp).

DNA ladder 100- 1.500 bp containing 11 fragments supplied in storage buffer 10mM Tris-HCL pH (7.5), 0.1 mM EDTA.

2.1.5.10. Protein electrophoresis by cellulose acetate paper (Dacie and Lewis, 1996).

The following buffers and solutions were used for electrophoresis by cellulose acetate paper

2.1.5.10.1. Barbitone buffer pH 8.6.

Sodium diethyl barbiturate	5.15 g
Barbitone (diethylbarbituric acid)	0.92 g
Distilled water	1000 ml

2.1.5.10.2. Staining solution.

Ponceau S	5 g
Trichloroacetic acid	7.5 g
Distilled water	1000 ml

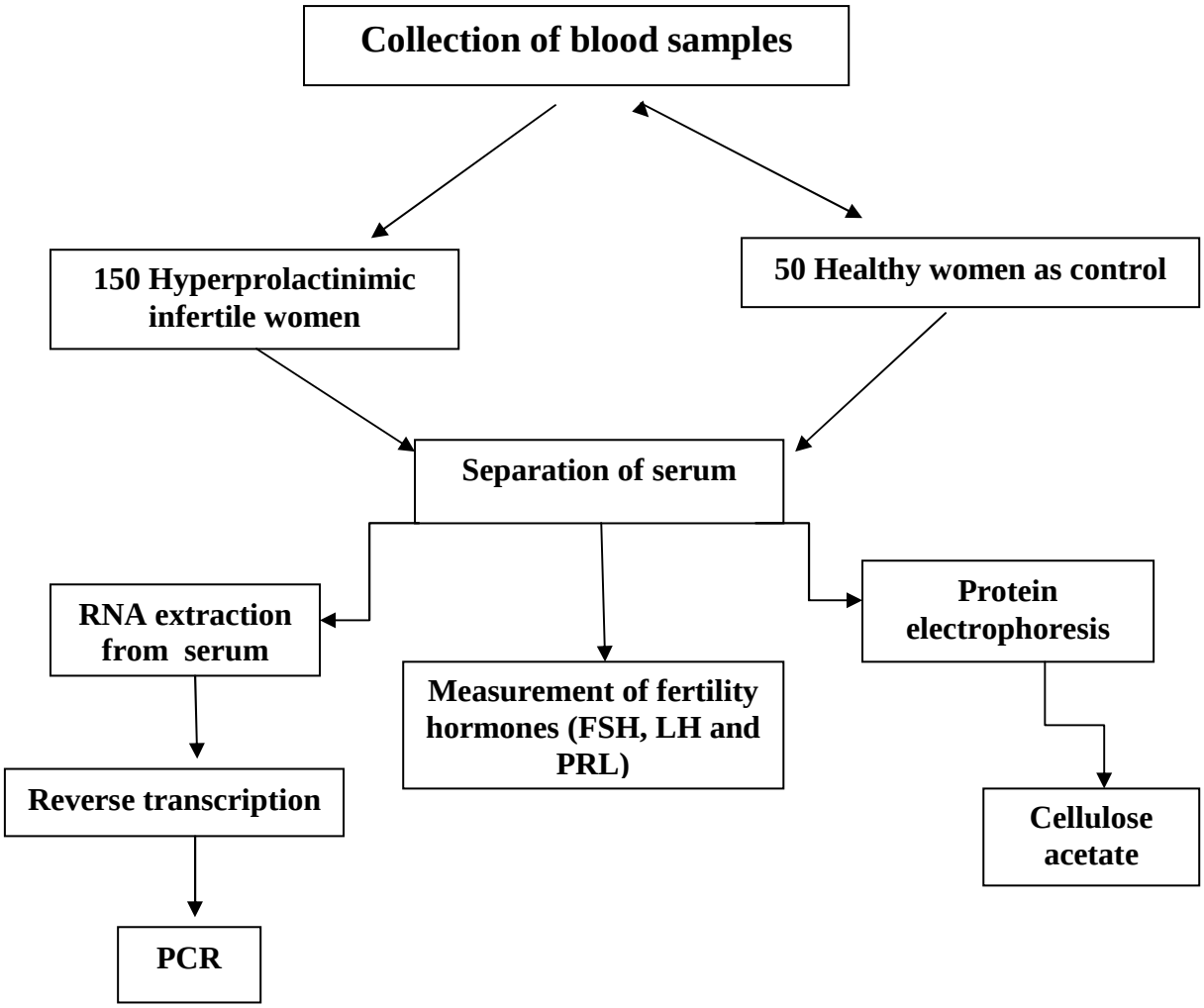
2.1.5.10.3. Destainig solution.

Acetic acid 5 % (v/v)	50 ml
Distilled water	1000 ml

2.1.5.10.4. Clearing solution.

Glacial acetic acid	125 ml
Methanol	375 ml
Polyethylene glycol	20 ml

2.2. Methods



2.2.1. Sterilization methods.

- Autoclaving: solutions were sterilized by pressure vessel (autoclave) at 121 ^{0}C and 15 bar for 15 minutes.
- Dry heat: A laboratory oven was used for glassware sterilization. Glassware was placed in the oven at 200 ^{0}C for 2 hours.

2.2.2. Determination of FSH, LH, and Prolactin levels (Butt and Blunt, 1988)

In FSH test the assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection (ELFA).

The solid phase receptacle (SPR), serves as the solid phase as well as the pipetting device for the assay. Reagents for the assay are ready to use and predispensed in the sealed reagent strips

All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times.

The sample is taken and transferred into the well containing alkaline phosphate- labeled anti – FSH (conjugate). The sample / conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to antibodies coated on the SPR and to the conjugate forming a “sandwich “.

Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4 – Methyl – Umbelliferyl phosphate) is cycled in and out of the SPR . The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescence of which is measured at 450nm. The intensity of the fluorescence is proportional to the concentration of antigen present in the sample.

At the end of the assay, results are automatically calculated by the VIDAS in relation to the calibration curve stored in memory, and then printed out.

All the previous steps were also applied LH and prolactin hormones.

2.2.3. RNA Purification Mini Kit.

2.2.3.1. Procedure:

The extraction was briefly carried out as follow:

- Aliquote of 100 µl of proteinase k (42.8 u/mg) was added to microcentrifuge tube containing 500 µl of the serum sample they mixed thoroughly by vortexing.
- Aliquot of 350 µl of FARB buffer (B-mercaptoethanol added) was added to the sample mixture and mixed vigorously by vortexing, then incubated at room temperature for 5 min.
- The sample mixture was transferred to a filter column set and centrifuged at 14,000 rpm for 2 min.
- The clarified supernatant was transferred from the collection tube to a new microcentrifuge tube and the volume of the clear lysate was adjusted, one volume of 70% ethanol was added to the clear lysate and mixed well by vortexing.
- The ethanol added sample (including any precipitate) was transferred to FARB mini column set and centrifuged at 14,000 rpm for 1 min. then the supernatant was discarded.
- Aliquot of 500 µl of wash buffer1 was added to FARB mini column and centrifuged at 14,000 rpm for 1min. then the supernatant was discarded.
- FARB mini column was washed twice with 700 µl of wash buffer 2 and centrifuged at 14,000 rpm for 1 min. then the supernatant was discarded.
- The column was dried by centrifugation at 14,000 rpm for additional 3 min.
- FARB mini column was placed in elution tube.

- Aliquot of 50 µl of Rnase- free water was added to the membrane center of FARB mini column and standing for 1 min.
- RNA was eluted by centrifugation at 14,000 rpm for 2 min.
- RNA was stored at -70c.

2.2.4. Determination of RNA yield and purity spectrophotometrically.

This was carried out using Nanodrop device that can measure RNA yield and purity in 260, 280 and 320nm.

2.2.5. Gel electrophoresis analysis RNA.

- 1.0% agarose / formaldehyde was prepared by mixing: 72 ml sterile deionized, and 1 gm agarose .
- The gel was heated, cooled to about 55^{o}C, 10 ml of 10X MOPS electrophoresis buffer then added to18 ml formaldehyde (37%) and 5µl of (10mg/ml) ethidium bromide were mixed with the gel.
- The gel was poured in clean glass mold (16X12X4 cm) and allowed to solidify at room temperature (25 ^{o}C).
- RNA samples were prepared by mixing one part RNA sample with two parts RNA sample buffer to total volume 10-30µl .These samples were heated to 65 ^{o}C for 5 minutes and then cooled on ice for two minutes . 2 µl of RNA loading buffer was added.
- The RNA samples were loaded, the gel was run at 5 v/cm. The electrophoresis was continued until bromophenol blue migrated at least 2/3 of the length of the gel.
- The gel was viewed under U.V. light using U.V. transilluminator.

2.2.6. Reverse Transcription System Kit.

2.2.6.1. Procedure:

The RT-PCR reaction was carried out following the user manual instructions:

- Aliquot of 1 µl of random primer was added to 4µl of RNA sample in a reaction tube.
- The RNA and primer mix were heated in 70^{o}C for 5 minutes and then they immediately chilled in ice water for at least 5 minutes, then they were centrifuged for 10 seconds in a microcentrifuge.
- In a sterile 1.5 ml micro-centrifuge tubes the reverse transcription reaction mix was prepared by combining the following:

Optimal values of various components in the standardization of RT-PCR reactions.

Component	Volume	Concentration
GoScript™ Reaction buffer	4 µl	5 x
$MgCl_2$	6.4 µl	5mM
PCR nucleotide mix	1 µl	0.5 mM
Ribonuclease inhibitor (20 unit/ µl)	0.5 µl	1 unit / µl
GoScript™ Reverse Transcriptase	1 µl	_
Nuclease Free Water	2.1	_
Total Volume	15 µl	_

- A volume of 5µl of RNA and random primer mix was added to final volume 15µl of reverse transcription reaction mix.
- The mixture was annealed at 25°C for 5 minutes and then they were extended at 42°C for one hour.
- The reverse transcriptase was inactivated at 70°C for 15 minutes.

2.2.7. PCR Amplification.

2.2.7.1 Procedure:

- The Go Taq® Master Mix was thawed at room temperature. The master mix was mixed by vortexing then it was spined briefly in a microcentrifuge.
- The reaction mix was prepared by combining the following:

Optimal values of various components of the amplification.

Components	Volume	Concentration
Go Taq® Green Master Mix	12.5 µl	1x
Upstream primer	0.5 µl	0.2 µM
Downstream primer	0.5 µl	0.2 µM
DNA template	5 µl	< 250 ng
Nuclease free water	6.5 µl	-
Final volume	25 µl	-

- The reaction mix was placed in thermal cycler using the protocol shown in table blew:

The Optimal protocol of PCR amplification.

Thermal cycler protocol	No. of cycles	Temperature/ Time
Initial Denaturation	1 cycle	94^{o} C for 2 min.
Denaturation	35 cycles	94^{o} C for 30 Second
Annealing		56.5^{o} C for 30 Second
Extention		72^{o} C for 1 min.
Final Extention	1 cycle	72^{o} C for 5 min.

- PCR products were analyzed by agarose gel electrophoresis using 2% of the total reaction mix.

2.2.8. Hot Start Green Master Mix.

2.2.8.1.Procedure:

- The Go Taq® hot start green Master Mix was thawed at room temperature. The master mix was mixed by vortexing then it was spined briefly in a microcentrifuge.
- The reaction mix was prepared by combining the following:

Optimal values of various components of the amplification.

Components	Volume	Concentration
Go Taq® Hot Start Green Master Mix	12.5 µl	1x
Upstream primer	0.5 µl	0.2 µM
Downstream primer	0.5 µl	0.2 µM
DNA template	5 µl	< 250 ng
Nuclease free water	6.5 µl	-
Final volume	25 µl	-

The reaction was placed in thermal cycler using the protocol shown in table blew

Optimal protocol of PCR amplification.

Thermal cycler protocol	No. of cycles	Temperature/ Time
Hot Start		95°C
Initial Denaturation	1 cycle	94° C for 2 min.
Denaturation	35 cycles	94° C for 30 Second
Annealing		56.5° C for 30 Second
Extention		72° C for 1 min.
Final Extention	1 cycle	72 ° for 5 min.

The PCR products were analyzed by agarose gel electrophoresis using 2% of the total reaction mix.

2.2.9. Reverse transcription (RT) PCR product gel electrophoresis (Maniatis *et al.* , 1982).

- 2% agarose gel was prepared by mixing: 100ml of 0.5X TBE buffer and 2 gm agarose in aglass bottle. A glass bottle was heated in a magnetic stirrer with heater until the agarose was dissolved.
- This solution was cooled to 70^{o}C, 5 µl ethidium bromide was added from stock solution and mixed thoroughly.
- The clean glass mold (17X12X4 cm) was set on a horizontal section of the bench. The comb was set in position 0.5-1.0 mm above the surface of mold so that a complete well was formed when agarose was added.
- The warm agarose – solution was poured into the mold.
- After the gel was completely set (20-30 min. at room temperature) the comp was carefully removed and the gel mounted in the electrophoresis tank which contain previously small amount of 1X TBE buffer.
- A volume of 600ml of 1X TBE was added to cover the gel in depth about 1mm.
- A volume of 10 µl of the sample of RT – PCR product was mixed with 2 µl of loading buffer. The mixture was set slowly into the slots of the submerged gel using an automatic micropipette.
- A volume of 5 µl of DNA marker was mixed with 1 µl of loading buffer. The mixture was set slowly into the slots of the submerged gel using an automatic micropipette.

- The gel was subjected to electrophoresis at 80 volts until the bromophenol blue tracking dye, migrated at least two-thirds of the way down the gel.
- The gel was examined by ultraviolet light using aUV transilluminator then photographed.

2.2.10. Protein electrophoresis.

2.2.10.1. Electrophoresis by cellulose – acetate paper (Daci and Lewis, 1996).

- The compartments of the electrophoresis tank were filled with barbitone buffer and the wicks were soaked and positioned in place.
- The cellulose paper was soaked in a separated dish in barbitone buffer for at least 5 minutes. The paper was immersed slowly to avoid trapping air bubbles and ensure even saturation of the membrane.
- The membrane was placed between two pieces of absorbent papers.
- A small volume (10 µl) of each diluted sample was placed into the sample well.
- The applicator was dipped into the sample wells, and the samples were applied to cellulose – acetate membrane approximately 3 cm from one end of the membrane. The tips of the applicator were allowed to remain in contact with the membrane for 3 seconds.
- The cellulose – acetate membrane was placed across the bridge of the tank so that the membrane surface is in contact with the buffer, with the line of the cathode end.
- The power supply was connected and run at 250 – 300 volt for 20 minutes or until a visible separation is obtained.
- The power supply was disconnected, and the cellulose – acetate membrane was removed and stained in ponceau S for 3 – 5 minutes.

- The membrane is then removed, drained, and the excess stain was eluted with three changes of destaining solution for 2 minutes each.
- The dehydration was performed in absolute methanol for 2 – 3 minutes.
- The membrane then was immersed in clearing solution for 6 – 8 minutes.
- After clearing the membrane it was dried at 65 ^{0}C for 4 – 6 minutes.

2.3. Statistical analysis.

Statistical analysis was done using Minitab 15 statistical analysis software. Two way ANOVA test was used to compare different groups among each other and with control. All values were expressed as Mean ± Standard Deviation of the mean (M ± SD). P value < 0.05 was regarded as statistically significant.

Chapter One

Introduction and Literatures Review

Chapter Two

Materials and Methods

Chapter Three

Results and Discussion

Chapter Four

Conclusions and Recommendations

References

Appendices

4. Conclusions and Recommendations.

4.1. Conclusions.

1- There is an association between gonadotrophin (FSH and LH) deficiency and hyperprolactinemia and this may be a sign of functional hypothalamic pituitary interruption due to the inhibitory effect of prolactin on gonadotrophin release.

2- Isolation of prolactin related RNA from serum may be considered as an indicator for the presence of a fault at the molecular level in prolactine gene.

Dedication

To those who are the beats of life in my heart

My family

To those who are the reason for my existence.

My Father and Mother

To the warm spring that floods my heart with love.

My sister Sarah

To those whom their suffering inspired me for this work.

The Patient's

To all those I dedicate this humble work.

Noor

List of abbreviations

ACTH	Adrenocortico tropic hormone
bb-PRL	Big-big prolactin
Bp	Base pair
CIS	Cytokine inducible SH-2 containing protein
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylendiaminetetra acetic acid
FSH	Follicle – stimulating hormone
GAS	Gama interferon activated sequence
GH	Growth hormone
Hox	Homeobox gene
ICSH	Interstitial cell stimulating hormone
Jak	Janus kinase
KD	Kilodalton
LH	Luteinizing hormone
MAPK	Mitogen activated protein kinase
MOPS	(3 – [N – morpholino] propan sulfuric acid)
MRI	Magnetic resonance imaging
mRNA	messenger Ribonucleic Acid
PCR	Polymerase chain reaction
PIH	Prolactin inhibition hormone

PRL	Prolactin
PRL-R	Prolactin receptor
RNA	Ribonucleic Acid
Rpm	Rotation per minute
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
SD	Standard deviation
SOCS	Suppressor of cytokine signaling
Stat	Signal transducer and activator of transcription
T3	Triiodothyronine
T4	Thyroxine
TRH	Thyrotropin releasing hormone
TSH	Thyroid – stimulating hormone
XFD	Xenopus fork domain related

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4.2 Recommendations

1-Performing DNA sequencing of prolactin promoter region to determine the transcription factor(s) that regulate the gene expression.

2-Real time quantitative PCR may be needed to detect the expression domain of prolactin gene.

3- For small DNA fragments resulted from cDNA amplification, a restriction analysis can performed for comparison between them.

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Supervisors Certification

We, certify that this thesis entitled **''Early Detection of Prolactin Elevation in Women Suffering from Infertility''** was prepared by **''Noor Ahmed Jihad''** under our supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

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الخلاصة

هذه الدراسة ركزت على موضوع يتعلق بحالات عدم الخصوبه التي سببها ارتفاع هرمون الحليب . حيث جمعت حوالي 150 عينة دم من نساء يعانن من ارتفاع هرمون الحليب اضافة الى ٥٠ عينة من نساء بحالة طبيعية استخدمت كمجموعة سيطرة. النساء اللواتي يعانن من المرض قسمت الى ثلاثة مجاميع عمريه : ٢٠-٣٠ سنة من العمر و ٣١-٤٠ سنة من العمر و ٤١-٥٠ سنة من العمر. تم اختبار وقياس مستوى هرمونات الخصوبة وهي هرمون الاباضة وهرمون المحفز للجريبات المبيضية وهرمون الحليب. لوحظ وجود فروقات معنويه $P<0.05$ في تراكيز الهرمونات عند مقارنتها بمجموعه السيطره حيث وجد ان مستويات هرموني الاباضة والمحفز للجريبات المبيضية قد سجلت انخفاضا معنويا واضحا بينما سجل هرمون الحليب ارتفاعا معنويا كبيرا عند مقارنة النتيجة مع مجموعة السيطرة.

لقد تم عزل الـــ RNA من مصل دم المرضى نتيجة ارتفاع مستوى التعبير الجيني لهرمون الحليب حيث تم تسجيل معدل تركيز قدره ٣٠٠ نانوغرام.

ان مستوى التحليل الجزيئي وباستخدام ثلاثة بادئات متخصصة صممت لهذا الغرض، وجد ان هنالك خلل في التعبير الجيني لهرمون الحليب في بعض المرضى بينما كانت مستقبلات هرمون الحليب طبيعية في المرضى ضمن هذه الدراسة.

استنتجت هذه الدراسة وجود خلل في ميكانيكية السيطرة الراجعه التي تسيطر على مستوى الهرمون في الجسم، اضافة ان هنالك بعض المرضى كانوا مرشحين لظهور سرطان الثدي بالاعتماد على تاريخهم العائلي وان معظم حالات ظهور هذا المرض كان ممتدا من اختلال في التعبير الجيني لهرمون الحليب.

التحليل البروتيني لمصل الدم للمرضى اظهر ارتفاعا معنويا في قيمة الالبومين والذي عزي الى ارتفاع هرمون الحليب حيث يلعب هذا البروتين دور الناقل للهرمونات في الدم.

جمهورية العراق
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كلية العلوم

التحري المبكر عن ارتفاع نسبه هرمون الحليب في بعض النساء اللاتي لديهن نقص في الخصوبه

رسالة

مقدمة الى كلية العلوم/جامعة النهرين

وهي جزء من متطلبات نيل درجة الماجستير علوم/ تقانة احيائية

من قبل

نور احمد جهاد

بكالوريوس علوم/تقانة احيائية/كلية العلوم /جامعة النهرين

(٢٠١٠-٢٠١١)

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