Expression level of FSH-r and LH-r genes in normal dominant and cystic ovarian follicles of local breed cows.

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Dedication

This thesis is dedicated to the soul of my father, symbol of love and kindness my mother who have supported me all the way since the beginning of my studies.

My wife the great source of motivation and inspiration.

My brother and my sister with love.

My dearly children (Bahaa, Ameen & Farah).

All those who believe in the richness of learning.

Alaa
Supervisors Certification

We certify that this thesis entitled "Expression level of FSH-r and LH-r genes in normal dominant and cystic ovarian follicles of local breed cows" was prepared under our supervision at the College of Veterinary Medicine, Al-Qadisiya University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Medicine/ Theriogenology.

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The recommendation of the Department

In the view of the available recommendation, I forward this thesis for debate by the examining committee.

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ABSTRACT

The present study was carried out to investigate the possible etiology and pathology of cystic ovarian follicles by determining the expression levels of mRNA for LH-r and FSH-r genes in follicular cells of dominant follicles in comparison with cystic ovarian follicles in Iraqi cows. Forty four ovaries were collected from sexually mature cows (4-6) years with unknown reproductive status from Al-Qadisiya, Al-Najaf and Karbala slaughterhouses during the period extended from December 2011 to October 2012, this study were performed in three steps, firstly aspiration of follicular fluids from dominant follicles and cystic follicles and stored in (-20°C) until estradiol and progesterone assay, secondary section of the follicular wall in to two hemispheres, one part for histological examination and the other part stored at -70°C to -80 °C to molecular study.

Macroscopic examination of the ovaries revealed that the numbers of dominant follicles are (23) samples while cystic follicles were in (21) samples. There was a significant difference (P<0.05) in the diameter of cystic ovarian follicles (37.56 ± 0.64 mm) compared with dominant follicles (19.93 ± 0.32 mm).

Results of hormonal assay showed higher estradiol-17β (865.96±10.64 ng/ml) and progesterone (84.8±1.35 ng/ml) concentrations in follicular fluids of cystic ovarian follicles, which were significantly higher (P<0.05) in comparison with those of dominant follicles which were 314.39±2.55 ng/ml and 50.25±1.57 ng/ml respectively.

Microscopic examination of the follicular wall showed significant difference (P<0.05) in the thickness of granulose cell layer of cystic ovarian follicles (57.69±2.38 µm), compared with that recorded in the dominant follicles (18.9±0.65 µm).
Molecular study, to evaluate the relative quantification of LH-r and FSH-r genes in dominant and cystic follicular cells, has been done by extraction of the total RNA and assay its concentration from these cells, synthesis data of the complementary DNA (cDNA), that done by reverse transcription PCR (q-RT-PCR) technique, of targets genes and compered of the gene expression in dominant and cystic follicular cells, showed down-regulation in the expression of FSH-r gene in the healthy dominant follicles, but it's expression were up-regulated in the cystic ovarian follicles, whereas expression level of LH-r gene was down-regulated in cystic ovarian follicles, and up-regulated in the follicular cells of dominant follicles.

In conclusion, our results referred to down regulation of LH-r gene expression in follicular cells of the cystic ovarian follicles, that may be assistance to understand the etiology and pathology of this case (disease), thus the up-regulation of the this gene in cells of dominant follicles may explain the important role of the LH in ovulation mechanism and increasing follicular ovulation chance. On the other hand, the up-regulation of FSH-r gene expression in these cells may explain the synergistic activity of estrogen and FSH which may leads to the cystic follicular development on the ovary.
Introduction

The local breed cow production could be described as multi-purpose providing milk, meat, clothing, fertilizer and calves. Despite this economic importance; the productivity of cows in Iraq is low due to the genetic potential of indigenous breeds, poor husbandry and a variety of environmental factors, including high ambient temperature and humidity, seasonal shortages of feed and water, reproduction diseases problems like cystic ovarian follicles (COF) which are considered as importance cause of subfertility in local cow, prolongation of the calving interval and treatment costs of COF result in economic losses for the farmer. The characteristic of COF is the presence of large, persistent, un-ovulatory follicles on the ovaries, with thin wall (Maniwa et al., 2005; Monniaux et al., 2008), and will be interferes with the estrous cycle (Wiltbank et al., 2002; Robert et al., 2005). The physiology and etiology of COF are poorly understood, however there is much conjecture regarding of the biological cause of COF, like altered of the pre-ovulatory LH surge from the hypothalamus-pituitary is either absent or insufficient during dominant follicle maturation, which leads to cystic formation (Gordon, 2002; Wiltbank et al., 2002; peter, 2004).

The follicular fluids contain high estradiol-17β and progesterone concentration in COF compared with the dominant follicles, yet the ratio of E2:P4 was greater than one (Silvia et al., 2002; Ball& Peter, 2004), so the steroid hormones in COF, particularly high concentrations of E2, would lead to genetic alterations, because the E2 inhibits P4 secretion.

The dominant follicle will produce sufficient E2 to induce a pre-ovulatory LH-surge, whereas the super basal concentrations of P4 blocks the pre-ovulatory LH-surge and lead to fail the new dominant
follicle to trigger a LH-surge and becomes cystic, while reduce of the P4 concentrations are likely to be associated with cyst turnover (Fortune et al., 2001; Beg & Ginther, 2006).

The granulosa layer is steroidogenic cells of the ovary, with the two layer theca interna and theca externa. These cell comprises a layer of ovarian follicles which appear as the follicles when become tertiary follicles and component of the follicular wall (Rodgers & Irving-Rodger, 2009; Paredes et al., 2011).

The granulosa and theca cells of COF relation with delays follicular regression, the granulose cells are distinguished by a high concentration of E2 and a low concentration of P4 in follicular fluids of COF (Grado-Ahuir et al., 2009), yet the granulosa cells are the main role to produce E2 and elimination of these cells will reduce the E2 concentration (Isobe & Yoshimura, 2007).

The FSH and LH are the necessary regulators of follicular maturation, because this two gonadotropins act by binding to and activating their specific receptors such as FSH-r and LH-r (Wiltbank et al., 2002).

The FSH-r exclusively are found on granulosa cells, while LH-r are found on theca cells during prenatal follicle development (Heckert et al., 2000; Dunn & Mayo, 2006), the genetic alteration effects on functioning of many cells kinds and/or tissues. However little is known about the genetic alterations that may be involved in pathogenesis of COF, the Gene expression comparisons may aid in understanding additional causes of COF, and will be vital to understand the entire process of ovulation failure and cyst formation.
Aims of the present study

The aims of the present study were:

1- Explain the relationship between COF occurrence and hormonal receptors production, especially FSH-r and LH-r.
2- Recognized the gene expression or mRNA quantification level of these two types of hormonal receptor genes (FSH-r and LH-r ) which are played an important role in reproduction, follicular development, ovulation and infertility.

2. Review of literature.
2.1. Definitions and Characteristics of Cystic Ovarian Follicle.

Cystic ovarian follicle (COF) a serious cause of the reproductive failure in cattle because they occur frequently and prolong the intervals from postpartum to first estrus and conception (Garverick, 1997).

COF is defined as ovarian structures, larger than a pre-ovulatory follicle (>25 mm in diameter) and persists for ten or more days in the absence of a corpus luteum (Gordon, 2002 and Youngquist & Threlfall, 2007).

Calder et al. (1999, 2001) and Fleischer et. al. (2001) reported that the (20 mm) diameter as a minimum size of the follicular cyst in cow, but Hatler et al. (2003) succeed that the follicles typically ovulate at 17 mm in diameter, while Vanholder et al. (2006) suggested that the COF should be defined as “follicles with a diameter of at least 20 mm that are present on one or both ovaries in the absence of any luteal tissue and that clearly interfere with normal ovarian cyclicity.

Leonardo & colin (2004) has been refer to many terms which used to describe the same condition are Cystic Ovarian Degeneration, Cystic Ovary, Ovarian Cysts, Cystic Cows and Nymphomania, but Vanholder (2005) used the
term COF to describe of this state “disease” which indicated to the ovarian follicle(s) and not any other ovarian tissue that becomes cystic.

COF results from a malfunction of the neuroendocrine mechanism controlling ovulation which developed when one or more follicles fail to ovulate and subsequently do not regress but maintain their growth and steroidogenesis with absence of luteal tissue, therefor this interferes with the estrous cycle (Arthur et al., 2001 and Wiltbank et al., 2002).

COF have been reported in many mammalian species, including rats (Brawer et al., 1986), dogs (Arbeiter, 1993), rabbits (Lopez-Bejar et al., 1998), swine (Heinonen et al., 1998), sheep (Christman et al., 2000), mares (McCue & Squires, 2002), cattle (Robert, 2004) and in the camel (AL-Delemy, 2007).

COF is a cause of temporary infertility, especially in the postpartum period. It is formation of a cyst after ovulation failure and causes ovarian dysfunctions (Hooijer et al., 2001 and Silvia et al., 2002).

COF characterized by thin wall and accumulation of an excess amount of follicular fluid (FF) inside the follicle which contains of many components, including hormones like estradiol-17β (E2) and very small amounts of progesterone (P4). The FF also contain of proteins like glyceraldehyde-3-phosphate dehydrogenase (GABDH) with its receptors (Ball & Peters, 2004; Isobe et al., 2005a; Maniwa et al., 2005 and Monniaux et al., 2008).

2.2. Histology of Follicular Wall to the Dominant and Cystic Follicle.

Histological notation on the granulosa layer is event out, to form together with the two layers, theca interna and theca externa, this cells which is component of the follicular wall (Arthure et al., 2001 and Irving Rogers et al., 2009).

2.2.1. Granulosa Cells.

Granulosa cells are steroidogenic cells of the ovary surround the oocyte, and enclosed in a thin layer of extracellular matrix which are follicular basement membrane or basal lamina (Turzillo & Fortune, 1993 and Evans &
Fortune, 1997), its cells may be seen in light microscope as simple columnar cells in growing follicles or in bunches in secondary or antral follicles as stratified cuboidal follicular epithelium cells (Ginther et al., 2001).

The major functions of this cells include the production of sex steroids by aromatase, during the follicular phase of the estrus cycle by responding to high levels of circulating gonadotropins (FSH) which stimulating the granulosa cells to convert androgens which coming from the thecal cells (Zimmermann et al., 2003 and Sasson et al., 2003), furthermore the maximum follicle stimulating hormone-receptor (FSH-r) and luteinizing hormone-receptor (LH-r) expression are a responsiveness to stimulation of the E2 synthesis (Calder et al., 2001 and Isobe et al., 2005a).

2.2.2. Theca Cells.

Theca cells comprise a layer of the ovarian follicles which appear as the follicles when become tertiary follicles and divided into two layers, the theca interna and the theca externa. This two layers develop only follicles that reach the stage of having a large antrum (Ginther et al., 2001 and Mokoto et al., 2009), while the theca externa deeply penetrated in the theca interna. This cells are responsible for the production of androstenedione, and indirectly the production of E2, with helping of the enzyme aromatase (Wandji et al., 1996; Gutierrez et al., 2000 and Braw-Tal & Roth, 2005).

2.3. Folliculogenesis in Cattle.

In cows, there are two or three waves of follicular development during an estrous cycle controlled by the neuroendocrine system, each wave consists of three to six follicles (>5) mm in diameter. After a several days of the emergence wave, one follicle becomes larger from others called dominant follicle (DF) which continues to grow (Hafez & Hafez, 2000; Ginther et al., 2001 and Mihm & Bleach, 2003).
The selection mechanisms of the DF are a complex and not fully understood, but the excessive secretion of E2 in FF of the future DF leads to increases blood estradiol concentrations (Hossner, 2005). This mechanism stimulation of gene expression for FSH and LH receptors in granulosa cells undergoes further maturation in preparation for ovulation. (Arthur et al., 2001; Johnson, 2003 and Senger, 2003).

Wiltbank et al. (2002) describe that the acquired of FSH-r and LH-r by the DF in response to LH and FSH are some of the many changes occurring with this follicles to preparing for ovulation, and (10–15)% of cases, this DF fails to ovulate and continues to grow into a COF.

Greenwald & Roy (1994) and Dierich et al. (1998) recorded that the growth of DF depends on FSH action, therefore lack of FSH function because of a null mutation in FSH-r gene which lead to incomplete of the follicular development.

Magoffin et al. (1990) and Zhang & Veldhuis (2004) mention that the exposed to relatively low levels of LH as follicle maturation proceeds and then to a single high-level surge of LH driven by hypothalamic release of gonadotropin-releasing hormone (GnRH) which induces ovulation and formation of the corpus luteum (CL).

Beg & Ginther (2006) indicate that the future DF acquired LH-r before follicular deviation, whereas others like Fortune et al. (2001) and Barros et al. (2009) reported that LH-r expression occurred after follicular deviation, yet confirmed that hypothesis of higher follicle diameters lead to increase the gene expression of LH-r in granulosa cells.

The ability of follicles to responsible for pre-ovulatory LH surge is depended upon the time induction of LH-r during follicular maturation. If too few receptors are available, then ovulatory failure may occur. (Arthur et al., 2001).
Growth of DF during the low FSH of ovarian follicular waves is hypothesized to be associated with decreased expression of the FSH-r gene but enhanced expression of LH-r genes in granulosa cells and of genes involved in proliferation and survival of theca and (or) granulosa cells. (Mihm et al., 2006).

2.4. Pathogenesis of the Cystic Ovarian Follicle.

The biological cause of COF like the altered of the pre-ovulatory surge from the hypothalamus-pituitary is either absent or insufficient in occurring at the wrong time during DF maturation, which leads to cystic formation (Gordon, 2002 and Wiltbank et al., 2002).

During follicular phase, the E2 causes of positive feedback effect on hypothalamic GnRH secretion that in turn increases FSH and LH releases (peter, 2004). The E2 induces the pre-ovulatory LH surge in cattle by increasing pituitary sensitivity to GnRH and then increasing LH release (Kesler & Garveric, 1982), yet the slight increases in LH pulse frequency promoted follicular growth and dominance which were associated with increased plasma estradiol. (Stock & Fortune, 1993).

When the E2 failed to induce a subsequent GnRH/LH surge, this lead to destine of follicles to form cysts. It follicle persistent with a larger diameter and a longer lifespan than normal lead to increased peripheral E2 concentrations (Noble et al., 2000; Hooijer, 2001; Silvia et al., 2002 and Gumen et al., 2002).

However the strong correlation between GnRH-r gene expression level and pituitary growth and development (AL-Ghreery, 2012), as well as dysfunction of the positive feedback mechanism of E2 on release of GnRH lead to change the function of the hypothalamic-pituitary-ovarian axis, even though the pituitary gland is still able to release LH because of hormonal imbalance (Stock & Fortune, 1993 and Duchens et al., 1994).

On other hand, the abnormal circulating of P4 level (intermediate concentrations) interferes with the hypothalamus, this leads to fail releasing of GnRH
surge in reposing to the E2 stimulus (positive feedback) and result in the formation of COF (Jeffcoate & Ayliffe, 1995; Noble et al., 2000 and Rhodes, 2003). So the low P4 concentrations lead to block the LH surge and doesn't cause ovulate of the DF (Gumen et al., 2002; Brito & Palmer, 2004 and Bartolome et al., 2005).

2.5. Main causes of Cystic Ovarian Follicle.

The factors that do predispose cows to develop COF have been suggested as genetics, hormonal imbalances, producing more milk, or exogenous factors (Fleischer et al., 2001 and Brito & Palmer, 2004), as well as the hormonal alterations during the final stages of follicle growth and maturation may trigger cyst formation (vanholder et al., 2006).

2.5.1. Heritability Factors.

The heritability is no clear indication which genes are involved in the pathogenesis of cysts according Calder et al. (2001) and vanholder (2005) But Garverick (1997) and Hooijer et al. (2001) conclude that the heritability factor for cystic ovaries determined by a genetic predisposition for COF incidence of cysts.

Heritability factors were rather low, being (7-12 %) (Hooijer et al., 2001), like the friesian cows are more susceptible to ovarian cysts than the local breed in Iraq (Majeed & Taha, 1990).

2.5.2. High Milk Production.

The relationship between the milk production and COF occurrence were directed by genetics factors (Hooijer et al., 2001), however the cows which genetically selected for producing more milk production will also be more likely
to develop multiple COF over their lifetimes (Lopez-Gaitus & Lopez-Bejar, 2002 and Brito & Palmer, 2004).

COF case does not develop during every lactation period or during every ovarian cycle, because the genetic predisposition for the development and indicating that the gene expression may gain functional importance (Uribe et al., 1995 and Zwald et al., 2004).

2.5.3. Season.

The studies of seasonal effect on COF incidence more variable, some studies were unable to correct any effect (Bartlett et al., 1986 and Hooijer et al., 2001), while others represented an increased incidence during fall-winter or in winter, because higher levels of nutrients, high protein feed and heavy grain in this month's (Kesler & Griverick, 1982; Grohn et al., 1990 and Arthur et al., 2001) or because its development in warm periods than during cooler seasons (López-Gaitus & Lopez-Bejar, 2002).

2.5.4. Stress Factors.

The COF is in the highest incidence at (61-90) days postpartum, according to the studying investigated in Iraq (Housain, 1996), because the stress factors were caused by postpartum diseases like postpartum uterine infections (Woolums & Peter, 1994a and Ribadu et al., 2000).

Kawet (2004) review that the stress is a possible cause for bovine follicular cysts, by stimulate of ACTH on adrenal gland to releases of cortisol which caused decrease of the LH-r contents of the follicle cells, consequently. Finally ovulation is blocked and the follicle becomes cystic, yet López-Gaitus & Lopez-Bejar, (2002) state that there are physiological stresses ex. Milk fever, early embryonic loss, retained placenta, metritis, twin birth and dystocia can cause COF.

2.5.5. Nutritional Status.
Diekman & Green (1992) and Garverick (1997) suggest that the feeding (moldy feed contained fungi *Fusarium spp.* ) which has estrogenic activity compounds, may play a role in occurs of COF disease in the cow. The incidence rates of COF were increased in cows which feeding of selenium/vitamin E deficient diet during the dry period (Harrison *et al.*, 1984), as well as the environmental factors including management such as prolonged low energy intake and low dry matter intake with high protein, lead to occurred of this case or disease (Vanholder *et al.*, 2006 and Simensen *et al.*, 2010).

**2.5.6. Age.**

COF effects on cows of all ages, but older cows have a higher incidence of ovarian cysts in (50%) over (11) years, yet it most commonly recognizes in cows with three to six years of age and increases of incidences in fourth or fifth lactation in dairy cows, and in beef cows after (4-6) years of age even (Robert, 2004).

**2.6. Clinical Signs.**

**2.6.1. Rectal Palpation Criteria.**

1. Formation of a COF or cysts fluid-filled ($\geq 25$) mm in diameter on the ovary or ovaries persists for a variable period in absence of a corpus luteum (Youngquist & Threlfall, 2007).

2. Relaxation of the sacrosciatic and sacroiliac ligaments with loss of muscle tone in the female genital tract (vulva, vagina, cervix and uterus) with passive prolapse of the vagina and excessive discharge of mucus (Johnson *et al.*, 1997 and Robert, 2004).

**2.6.2. Behavioral Changes.**

1. Prolonged estrual behavior termed nymphomania which characterized by excessive mounting, standing, bawling with noticeably deeper tone and erratic milk production, resulting from low P4 due to the absence of a functional CL
and increased E2 from the cystic follicle (Youngquist, 1986 and Robert, 2004).

2. Sterility hump appearance of the tail head is resulting from relaxation of sacroiliac and sacrosciatic ligaments of the pelvis (Garverick, 1997 and Johnson et al., 1997).

2.7. Diagnosis.

Rectal palpation was highly inexact in diagnosing cyst type (COF, luteal cyst and cystic of corpus luteum), the indication of COF by present of greater follicles structure filled with large amount of FF, although false positive diagnoses may be made in approximately (10%) (Farin et al., 1992 and Hatler et al., 2003).

Diagnosis of an enlarged ovary is fairly straight forward by palpation, but distinguishing a follicular from a luteal cyst, or determining presence of several structures, is less accurate, and its correctly diagnosed the presence and type of cyst in (41-85)% of cases (Douthwaite & Dobson, 2000 and Peter, 2004).

The circulating P4 concentrations analysis to improve the diagnosis of COF, by definition serum P4 concentration is (<1 ng/ml) in cases of COF (Hatler et al., 2003).

The trans-rectal ultrasonography which are more reliable methods to identification of COF by used since ovarian structures can be visualized and true cysts can be differentiated from other ovarian structures. (Gordon, 2002; Senger, 2003 and Bartolome et al., 2005).

The ultrasonic imaging is a highly accurate and rapid method of assessing ovarian structures (Griffin & Ginther, 1992 and Fricke, 2002), figure (2-1).
2.8. Steroidal Hormones in Follicular Fluid of the Dominant Follicles and Cystic Ovarian Follicles.

The follicle health and steroidogenic status of the follicular cyst were determined by measurement of the E2 and P4 concentration in follicular fluid (FF) (Badinga et al., 1992 and Landau et al., 2000).

DF has elevated E2 concentrations in the FF which may increase the responsiveness of the DF to gonadotropin stimulation, but the COF is produces a high concentration of E2, because the prolonged growth of follicles which lead to increased follicular E2 synthesis (Hafez & Hafez, 2000 and Calder et al., 2001), yet the DF has the capacity to produce large amounts of steroids, especially the E2 and low amount P4 (Sirois & Fortune, 1990; Hamilton et al., 1995 and Yoshioka et al., 1996).

COF had E2 concentration in FF varied and originates from granulosa cells, therefore the level of E2 concentration is an important indicator to determine the stage of COF (Isobe et al. 2005a).
2.9. The Necessary Receptors for Follicular Maturation and Ovulation.

2.9.1. Follicle Stimulating Hormone–Receptors.

The structures of follicle stimulating hormone-receptors (FSH-r) are transmembrane proteins (G protein-coupled receptors) composed of seven transmembrane (Congreve & Marshall, 2010), that interacts with the Follicle Stimulating Hormone (FSH), and become active when the FSH binding to this receptors (Gromoll et al., 1996; Ryu et al., 1998 and Schmidt et al., 2001). In cow, this receptors localized in the ovary only on the granulosa cells (Griswold et al., 1995 and Hunzicker-dunn & Mayo, 2006).

FSH binding externally with FSH-r on the cell membrane, a transduction of the signal that activates the G protein that is bound to the receptors internally takes place, then the G protein detaches from the receptors and activates the cyclic adenosine monophosphate (cAMP) system, the DNA in the cell nucleus binds to phosphorylated proteins which results in the activation of genes (Simoni et al., 1997 and Hunzicker-dunn & Mayo, 2006).

The expression of FSH-r (number of receptor sites on the membrane) is increased by FSH activation (Nakamura et al., 1995 and Minegishi et al., 2000), therefore enhance FSH action in the follicles by increasing FSH-r expression, yet the FSH-induced the granulosa cell aromatase activity and follicular growth (Yada et al., 1999).

2.9.2. Luteinizing Hormone-Receptors.

Luteinizing hormone-receptors (LH-r) like FSH-r which belongs to the superfamily of G-protein coupled receptors, structural from seven
transmembrane helix contains of extracellular domain, that receptors interacts with the luteinizing hormone (LH), and necessary for the activation of LH functioning (Dufau, 1998 and Huhtaniemi, 2000).

LH-r in female mammals is expressed in the ovary in theca cells of ovarian follicles, in granulosa cells of mature preovulatory follicles, and in corpora luteum. Its expression requires appropriate hormonal stimulation like E2 and FSH in preparation for ovulation. After ovulation, the luteinized ovary maintains LH-r that allows activation in case of there is an implantation. (Ascoli et al., 2002), yet the E2, and the LH-r are involved in intrafollicular events that initiate this divergence (Ginther et al., 2001 and Beg et al., 2001).

When LH interacts with LH-r will be influences various activities such as steroidogenesis, follicular growth, oocyte maturation, ovulation and corpus luteum formation, which are essential for reproductive function of the females (Hyttel et al., 1997). Therefore under physiologic conditions, the appearance of LH-r on granulosa cells is important for folliculogenesis from the acquisition of follicular dominance until ovulation (Ginther et al., 2001; Sartori et al., 2001 and Barros et al., 2010).

**FSH-r and LH-r Genes.**

The FSH-r and LH-r genes a relatively large genes and consists of ten exons and nine introns in all species, the single copy gene is characterized by the common structural features of seven transmembrane domains with six or seven amino acids in the C-terminal region of the third cytoplasmic loop implicated in G-protein coupling with ten exons and nine introns (Simoni et al., 1997; Hossner, 2005 and Hunzicker-dunn & Mayo, 2006).
2.9.3. FSH-r and LH-r mRNA Expression in Follicular Cells of Dominant and Cystic Ovarian Follicle.

FSH and LH are necessary regulators of follicular maturation, this is two gonadotropins act by binding and activating their specific receptors like FSH-r and LH-r, the FSH-r are exclusively found on granulosa cells and LH-r are found exclusively on theca cells during prenatal follicle development (Gromoll et al., 1996; Heckert et al., 2000 and Hunzicker-dunn & Mayo, 2006).

FSH-r gene is not expressed until follicles have reached to the pre-ovulatory stage in DF, this timing of the onset of FSH-r gene expression which are detect by reverse transcriptase quantitative real time-polymerase chain reaction (rt-PCR) is often the method of choice (Elvin & Matzuk, 1998 and La Marca et al., 2005).

LH-r in female mammals is expressed in theca and granulosa cells of mature pre-ovulatory follicles which requires hormonal stimulation by FSH and E2. The action of LH-r is necessary for folliculogenesis from follicular dominance until ovulation and luteal function (Sartori et al., 2001; Ascoli et al., 2002 and Barros et al., 2010).

The up-regulation of expression of genes for LH-r in granulose cells and down-regulation of expression of genes for FSH-r were associated with Growth of follicles until become of graafian follicles (Zhenzhong et al., 1995; Beg & Ginther, 2006).

2.10. Real Time Polymerase Chain Reaction.

Real-Time Polymerase Chain Reaction (rt-PCR) also called quantitative (rt-PCR) is a recent modification to PCR, It was first introduced by Higuchi et al. (1993), which used this technique to amplify and simultaneously quantify a targeted DNA molecule (Pfaffl, 2001 and Van-Guildet et al., 2008).

Nucleic acid RNA is the target of detection in real time reverse transcriptase PCR. This nucleic acid cannot serve as a template for PCR, so the
first step in an PCR assay is the Reverse Transcription of the RNA template into complementary DNA (cDNA), (Dorak, 2006).

There are many studies like Ginzinger (2002) and Robert et al. (2005) used this technique to explained the gene expression levels and measured the mRNA transcript levels in a quantitative fashion by combining the two technologies real-time PCR with reverse transcription (real-time -PCR).

Two different methods of analyzing data from q (rt-PCR) experiments exist: absolute quantification (Absolute standard curve method) and relative quantification (fold change) (Livak & Schmittgen, 2001). The relative quantification describes the change in expression of the target gene relative to some reference gene group such as (GAPDH) in eukaryotic cells to normalizing q(rt-PCR) data (Kreuzer et al, 1999 and Winer et al, 1999), yet there are three ways to application of the relative quantification methods like Livak method ($2^{\Delta \Delta Ct}$ method), $\Delta Ct$ method by using the reference gene, and Pfaffl method (Livak and Schmittgen, 2001 and Pfaffl, 2001).

Advent of quantitative rt-PCR techniques for the measurement of gene expression has allowed the accurate determination of the expression levels of target genes in cells and tissues (Robert et al., 2005), whereas the major disadvantage to real-time PCR is that it requires expensive equipment and reagents, due to its extremely high sensitivity experimental design and a depth understanding of normalization techniques are imperative for accurate conclusions (Wang & Brown, 1999).

Yin et al. (2001) and Dorak (2006) explain that rt-PCR chemistries consist of special fluorescent dyes (DNA-binding dyes) which binds nonspecifically to double-stranded DNA (ds DNA) SYBR Green I exhibits little fluorescence when it is free in solution.

Prevost et al.(2007) observed that the advantages of using SYBR Green I fluorescence dye like:
1. Its rapid and sensitive method to detect low amounts of mRNA molecules.
2. These dye important physiological insights on mRNA expression level.
3. Simple assay design.
4. Lower initial cost.

At the same time Mackay et al. (2002) and Prevost et al. (2007) revealed the main disadvantages such as:
1. Lack of specificity, this is DNA-binding dyes bind to any ds DNA including non-specific primer-dimers, as a result of the presence of the nonspecific products in a real-time PCR reaction which may contribute to the overall fluorescence and affect the accuracy of quantification.

2. The higher concentrations of SYBR Green I lead to inhibition of PCR reaction.

**2.10.1. Definition Some Terms Used in Real Time-PCR.**

1. **Threshold Cycle (Ct).**

   The cycle number or threshold cycle (Ct) are value measured in the exponential phase, used to reliably and accurately calculate the initial amount of template present in the start of the amplification reaction (Giulietti et al., 2001). If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background, while Ct values decrease linearly with increasing target quantity (Bustin & Nolan, 2004 and Jeanette & Jaime, 2011).

2. **Primer Design**

   A number of free and commercially available software programs are available for this purpose, one popular web-based program for primer design is Primer-3 [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi) (Bustin, 2000), Primers should bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. It is necessary to treat the RNA
sample with RNase-free DNase to minimize any problems caused by amplification of re-annealed DNA fragments (Bustin, & Nolan, 2004).

3. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH).

GAPDH (housekeeping gene) is an enzyme often stably and constitutively expressed at high levels in most tissues and cells, its commonly used by biological researchers as a loading control for rt-PCR (Bustin, 2000 and Robert et al., 2005). The idea of a housekeeping gene is that normal levels for the expression of GAPDH mRNA are defined, and clear differences are observed between different tissue types and no effects of age or gender were detected on the expression, therefore, these data provide standard values for levels of GAPDH mRNA expression in the tissues studied (Gorzelniak et al., 2001 and Bar et al., 2009).

2.10.2. Amplification Plot.

The fluorescence from the amplification reaction plot consists from two axes, the y-axis which is proportional to the amount of amplified product in the reaction tube, and the x-axis which represents the PCR cycle number (Von-Ahsen et al., 1999 and Bustin, 2000), this amplification plot divided in to three phases, figure (2.2).

A: Linear Phase (Early Exponential Phase): During the linear ground phase (usually the first 10–15 cycles), PCR is just beginning, and fluorescence emission at each cycle has not yet the fluorescence remains at background levels, (cycles 1–18) even though product accumulates exponentially (Tichopad et al., 2003).

The Ct value is representative of the starting copy number in the original template and is used to calculate experimental results (Heid et al., 1996 and von-Ahsen et al., 1999). If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background, but when the reaction will have a low (early) Ct in contrast, if a small amount of template is
present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background (Marisa & Juan, 2005).

**B: Exponential Phase:** which is describe for amount of PCR product approximately doubles in each cycle. The reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase (cycles 28–40) (Heid et al., 1996).

**C: Plateau Phase,** in this phase the reaction will slowdown and stop due to depletion of substrates and product inhibition (Bustin, 2000).

---

**Figure (2-2):** The PCR amplification curve, the cycle number (Ct) is shown on the x-axis, and the fluorescence from the amplified production on the y-axis (Prada-arismendy & Castellanos, 2011).

3.1. Materials.

3.1.1. Instruments.

Table (3.1): The instruments which used in this study with their companies and countries of origin.

<table>
<thead>
<tr>
<th>No.</th>
<th>Instruments</th>
<th>Company</th>
<th>country</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Autoclave</td>
<td>Tomy Vertical Autoclave</td>
<td>Germany</td>
</tr>
<tr>
<td>2</td>
<td>Cold Eppendrof centrifuge</td>
<td>Hermile Labortechnik</td>
<td>Germany</td>
</tr>
<tr>
<td>3</td>
<td>Deep Freeze</td>
<td>Gesellschaft Fur Labortechnik (GFL)</td>
<td>Germany</td>
</tr>
<tr>
<td>4</td>
<td>Desktop centrifuge</td>
<td>Hettich</td>
<td>Germany</td>
</tr>
<tr>
<td>5</td>
<td>Digital camera</td>
<td>Sony</td>
<td>China</td>
</tr>
<tr>
<td>6</td>
<td>Exispin(Vortex Centrifuge)</td>
<td>Bionner</td>
<td>Korea</td>
</tr>
<tr>
<td>7</td>
<td>Freezer</td>
<td>Concord</td>
<td>Lebanon</td>
</tr>
<tr>
<td>8</td>
<td>Gama Counter</td>
<td>Berthold</td>
<td>Germany</td>
</tr>
<tr>
<td>9</td>
<td>Gel electrophoresis apparatus</td>
<td>Consort</td>
<td>Belgium</td>
</tr>
<tr>
<td>10</td>
<td>Hot magnetic stirrer</td>
<td>Hysc</td>
<td>Korea</td>
</tr>
</tbody>
</table>
### 3.1.2. Equipment.

**Table (3.2): The equipment which used in this study with their companies and countries of origin.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Equipment</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cool box</td>
<td>Qmex</td>
<td>India</td>
</tr>
<tr>
<td>2</td>
<td>Cotton Buds</td>
<td>Lara</td>
<td>Turkey</td>
</tr>
<tr>
<td>3</td>
<td>Digital Verniaer</td>
<td>Reshan</td>
<td>China</td>
</tr>
<tr>
<td>4</td>
<td>Latex gloves without powder</td>
<td>Great</td>
<td>Malaysia</td>
</tr>
<tr>
<td>5</td>
<td>Micro centrifuge tubes 1.5 ml</td>
<td>Eppendrof</td>
<td>Germany</td>
</tr>
</tbody>
</table>
3.1.3. Chemicals.

Table (3.3): The chemicals which used in this study and their sources.

<table>
<thead>
<tr>
<th>No.</th>
<th>Chemicals</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acidic alcoholic eosin</td>
<td>Riedel-dehaenag</td>
<td>Germany</td>
</tr>
<tr>
<td>2</td>
<td>Agarose</td>
<td>Promega</td>
<td>USA</td>
</tr>
<tr>
<td>3</td>
<td>Chloroform</td>
<td>Labort</td>
<td>India</td>
</tr>
<tr>
<td>4</td>
<td>DEPC water*</td>
<td>Bioneer</td>
<td>Korea</td>
</tr>
<tr>
<td>5</td>
<td>EDTA</td>
<td>Sigma-Aldrich</td>
<td>USA</td>
</tr>
<tr>
<td>6</td>
<td>Eosin</td>
<td>Merk</td>
<td>Germany</td>
</tr>
<tr>
<td>7</td>
<td>Ethanol 1000%</td>
<td>Labort</td>
<td>India</td>
</tr>
<tr>
<td>8</td>
<td>Ethanol 80%</td>
<td>Invitrogen</td>
<td>India</td>
</tr>
</tbody>
</table>
### Table of Materials

<table>
<thead>
<tr>
<th>No.</th>
<th>Material Description</th>
<th>Supplier</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Ethidium bromide</td>
<td>Labort</td>
<td>USA</td>
</tr>
<tr>
<td>10</td>
<td>Formalin 10%</td>
<td>Labort</td>
<td>India</td>
</tr>
<tr>
<td>11</td>
<td>Hematoxylin</td>
<td>Merk</td>
<td>Germany</td>
</tr>
<tr>
<td>12</td>
<td>Isopropanol</td>
<td>Labort</td>
<td>India</td>
</tr>
<tr>
<td>13</td>
<td>Normal saline</td>
<td>Labort</td>
<td>India</td>
</tr>
<tr>
<td>14</td>
<td>Loading day</td>
<td>Labort</td>
<td>USA</td>
</tr>
<tr>
<td>15</td>
<td>Paraffin wax</td>
<td>Merk</td>
<td>Germany</td>
</tr>
<tr>
<td>16</td>
<td>PBS×10 solution**</td>
<td>Bio Basic</td>
<td>Canada</td>
</tr>
<tr>
<td>17</td>
<td>RNase free water***</td>
<td>Bioneer</td>
<td>Korea</td>
</tr>
<tr>
<td>18</td>
<td>Xylin</td>
<td>Milpharm</td>
<td>England</td>
</tr>
</tbody>
</table>

* DEPC-treated water/ (diethyl pyro carbonate).

**PBS×10 solution/PH (7.4-7.6) contains 137 mM (mile mole) Sodium Chloride and 10 mM Phosphate Buffer.

***RNase-free water / (third distilled water), filtered with 0.2 μm pore size.

### 3.1.4. Quantitative Reverse Transcriptase Real-Time PCR Kits.
Table (3.4): All kits which used in quantification of gene expression levels by qRT-PCR, and hormonal kit which used to RIA method, with their companies and countries of origin.

<table>
<thead>
<tr>
<th>No.</th>
<th>Kit</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AccuZol™ Total RNA Extraction Kit - Trizol 100ml</td>
<td>Bioneer</td>
<td>Korea</td>
</tr>
<tr>
<td>2</td>
<td>AccuPower® RockScript RT PreMix</td>
<td>Bioneer</td>
<td>Korea</td>
</tr>
<tr>
<td></td>
<td>- RocketScript Reverse Transcriptase (200 u)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 5× Reaction Buffer (1×)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- RNase Inhibitor (1 u)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- DTT (0.25 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Dntp (250 µM each)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AccuPower® Greenstar™ qPCR PreMix</td>
<td>Bioneer</td>
<td>Korea</td>
</tr>
<tr>
<td></td>
<td>- SYBER Green fluorescence</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Exicycler™ 20 µL reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 8 Well strips × 12 each</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- DEPC – D.W. 1.8 ml × 4 tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>EZ-10RNA Mini-Preps Kits Handbook</td>
<td>Bio basic</td>
<td>Canada</td>
</tr>
<tr>
<td></td>
<td>- RNase-Free DNase Set</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Immunotech RIA Progesterone (kit)</td>
<td>Beckman</td>
<td>France</td>
</tr>
<tr>
<td></td>
<td>- Coulter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Immunotech RIA Estradiol (kit)</td>
<td>Beckman</td>
<td>France</td>
</tr>
<tr>
<td></td>
<td>- Coulter</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.1.5. Primers.

Three set of primers are used in this study, first primer used for GAPDH gene as Housekeeping gene and other primers used for FSH-r gene and LH-r as target genes. These primers were designed by using NCBI-Gene Bank data base and Primer 3 design online, the primers used in quantification of gene expression using quantitative (real time-PCR) techniques based SYBER Green DNA binding dye, and supported from (Bioneer, Korea) company.

Table (3.5): The Primers with their sequences and product size.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Product size (Base Par)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABDH- forward</td>
<td>5'-AGCAACAGGGTGTTGGGAGCT-3'</td>
<td>133</td>
</tr>
<tr>
<td>GABDH- reveres</td>
<td>5'-AGTGTGGCGAGATGGGGCA-3'</td>
<td></td>
</tr>
<tr>
<td>FSHR-forward</td>
<td>5'-CCACGTGATGCGCGTCAGA-3'</td>
<td>360</td>
</tr>
<tr>
<td>FSHR- reveres</td>
<td>5'-GCTCCCTGAGCACAGCA-3'</td>
<td></td>
</tr>
<tr>
<td>LHR- forward</td>
<td>5'-CCGGAAGGGCCTGTTGCTCAT-3'</td>
<td>680</td>
</tr>
<tr>
<td>LHR- reveres</td>
<td>5'-GCGTCACCTCCGGGCAT-3'</td>
<td></td>
</tr>
</tbody>
</table>

3.2.1. Collection of the Follicles.

The presence study depends on collection of (44) ovaries from sexually mature cows (4-6 years) old with unknown reproductive status from Al-Qadisiya, Al-Najaf and Karbala slaughterhouses during December 2011 to October 2012, these ovaries were transported within 1-2 hrs. in cool box (ice) to the vet. laboratory of Al-Najaf hospital and freed from the surrounding tissue and over bursa, then each ovaries was subjected to washing in phosphate buffer saline (PBS) and one washing in ethanol 70%, examined by macroscopic exam (color, consistency, corpus luteum (CL) stage, follicular numbers and follicular size) according to Stock & Fortune (1993) and Berisha, et al. (2000a). Its ovaries were divided into two groups' dominant follicles (DF) and cystic ovarian follicles (COF) were used in this study.

A- Dominant Follicles

The dominant follicles (DF) group are (23) samples with diameter range 17-25 mm, contain only healthy follicles which having transparent fluids, signs of mucus production in the uterus and cervix and present of regress corpus luteum for previous estrus cycle, figure (3.1).

B- Cystic Ovarian Follicles.

The cystic ovarian follicles (COF) are (21) samples having large un-ovulated persistent follicles on the ovary and diagnosed by the macroscopic notation such as the follicle diameter are greater than 25 mm, absent of any corpus luteum in both the right and left ovaries and the follicular walls of the cysts were thin and translucent, figure (3.2).
Figure (3.1): Genitalia of different cows, showed the diameter of dominant follicle on the ovary; A. Follicle (20.05 mm) on lift ovary; B. Follicle (20.47 mm) on lift ovary; C. Follicle (18.00 mm) on lift ovary with presents regress CL of previous estrus cycle; D. Follicle (20.25 mm) on right ovary with presents regress CL of previous estrus cycle.
Figure (3.2): Genitalia of different cows, showed the diameter of cystic follicle on the ovary; A. Follicle (39.21 mm) on right ovary; B. Follicle (31.754 mm) on left ovary; C. Follicle (42.5 mm) on left ovary; D. follicular wall and follicular fluids (28 ml) of cystic follicle.
3.2.2. Study Design.

These two groups DF and COF have been done in three steps which are:

First step: Aspiration of follicular fluids (FF) from DF and COF and stored in -20°C until estradiol 17β (E2) and progesterone (P4) assay by used the radio-immunoassay (RIA) method.

Second step: Section of the follicular wall in to two hemispheres and place one of them in small container in formalin (10%) for histological examination.

Third step: The other part stored in free RNase eppendorf tubes (1.5 mm), and stored at -70°C to -80°C until RNA extraction and finally rt-PCR data analysis were undertaken (Relative quantification), diagram (3.1).

3.2.3. Estradiol-17β and Progesterone Assay in Follicular Fluid.

The FF was aspirated from DF and COF by 18 gage needle attached to 10 ml size disposable syringe which was inserted into follicular cavity, and collected separately in test tubes contain anticoagulant, then centrifugation 2000 rpm for 10 minutes and stored at -20°C until hormonal assay, as a described previously by Langhout et al. (1991); Vanholder (2005) and Aad et al. (2006), figures (3.3-A, B).

* Radio-immunoassay method.

The radio-immunoassay method (RIA) were done according to Adeyemo (1984) through following steps.

1. Add 50 µl of follicular fluid in anti-progesterone antibody-coated tubes, or (anti- estrogen antibody-coated tubes).

2. Add tracer (labeled progesterone) or (labeled estrogen) 500 µl.
3. Incubated this mixture for three hours in water bath at 18-25°C, with shaking system in 350 shocks per minutes.

4. Infuse of the tube contained which not binding with antibody.

5. Account of the antibody which contacted with sample for each minuet, count per minutes (CPM) bound by gamma-count depending on standard curve which specific to progesterone or (estradiol hormone).

3.2.4. Histological Study.

Preparation of the samples to histological sectioning according to Al-Attar et al. (1982) and Noory (1989) methods as the following:

1- Fixative of the follicular wall done by using of Formalin (10% ) to (24-48) hrs.

2- Washing the samples in water for three hrs. to remove the formalin residue , then the samples entered to a graded series of increasing ethanol concentration which were (70%, 80%, 90%, 95%) and (100)% for about 1-2 hrs. for each concentration .

3- Clearing by xylene for three times to 1-1.5 hrs.

4- Embedding by liquid paraffin in 56-58 °C for two times, then pouring in wax template.

6- Section the samples by the rotary microtome to 5 μm thickness and transported to water bath 50-55 °C and put it on slides which painted with mayor albumin.

7- De-paraffinization of slides by putting them in oven 55-70 for 5 hrs. then put them in xylene for one hour.
8- Dry it from xylene, then washed with water and put the slides in harris-hematoxyylene stain for 15 minutes and washing with flow water.

9- Put it in acidic alcohol for few second, and washing with water until return of blue color.

10- Put it in watery eosin stain for 10 min. and washing with flow water.

11- Put it in xylene for 24 hrs. and mounting the slides with the sticky material and cover slipping with slides covers and leave it to dry and examine of this sample by used the microscope with camera to determine the granulosa cell layer thickness by optical measure in the microscope lens.

3.2.5. Molecular analysis.

3.2.5.1. Isolation of the Follicular Cells.

Isolation of the follicular cells from the follicular frozen wall part (dominant or cyst) which are comprise from granulosa and theca cells (externa & interna) were collected by two experiments:

First Experiment:

According to Isobe et al. (2008) and Beg et al. (2001).

1- Weight 100 mg of frozen follicular wall.

2- Application of the homogenizer by mortar and pestle with liquid N2 (-196°C).

3- Collection of this mixture in numbered free RNase eppendorf tubes (1.5 mm).

4- Centrifugation 12000 rpm for 10 min at 4°C to isolated the pellets.
5- The follicular cell mashed were stored at -70 to -80°C in deep freeze system until total RNA extraction according to manufacturer's instructions of the AccuZol® Total RNA Extraction Kit.

Second Experiment:

Depended on study by Nogueira et al. (2007).

1- Weight 100 mg of frozen follicular wall and placed in a petri dish.
2- Gently scraped of internal layer using a plastic knife to isolate the follicular cells.
3- Flushed this piece with PBS to remove remaining follicular cells.
4- The solution was collected in 1.5 ml tube per dominant or cystic samples then centrifugation 12000 rpm for 10 minutes at 4°C.
5- Re suspended the sediment in numbered 1.5 ml tubes.

6- The follicular cell mashed was stored at -70°C to -80°C in deep freeze system until total RNA extraction, figures (3.3-C, D, E and F) showed the previous steps.
Figure (3-3): Collected of follicular cells from follicular walls,

A. Gentle aspirated of the follicular fluid, B. Collected of the follicular fluid separately in Venoject® tubes, C. Follicular wall was hemisphere in a Petri dish, D. Gently scraped of internal layer with the aid of a plastic knife to remove the follicular cells and flushed with PBS, E. Aspiration the result solution by used a syringe fitted with an 18-gauge needle, F. Collected this mashed in free RNase eppendorf tubes (1.5 ml).

3.2.5.2. Total RNA Extraction.

The total RNA was extracted from a follicular cell pellet using of the total RNA extraction reagent (Accuzol® Usere manual, BIONEER-Korea) and according to the manufacturer’s instructions, breviary description.
1- Thawing the frozen knlhcells mashed and application of the homogenizer by tip of the pipet with 1ml Accuzol®.

2- Add 200μl of chloroform per 1 ml of Accuzol® and shake vigorously for 15 seconds.

3- Incubate the mixture on ice for 5 minutes.

4- Centrifuge at 12000 rpm for 15 minutes at 4°C.

5- Transfer the aqueous phase to a new 1.5 ml tube and add equal volume of isopropanol.

6- Mix by inverting the tube 4-5 times and incubate at -20°C for (10) minutes.

7- Centrifuge at 12000 rpm for 10 minutes at 4°C, then carefully remove the supernatant (aqueous phase: total RNA).

8- Add 1 ml of Ethanol (80 %) and mix well by vortex.

9- Centrifuge at 12000 rpm for 5 minutes at 4°C, then carefully remove the supernatant.

10- Dry the pellet.

11- Dissolve RNA in RNase- free water by passing the solution a few times through a pipette filter tip, and store at -70°C to -80°C.

3.2.5.3. Quantification of total RNA.

Quality control standards were applied to all RNA samples in this study, these were that the purity was 1.7-1.9, total RNA samples were adjusted at same
concentrations. This is performing by nanodrop spectrophotometer machine (OPTIZEN POP. MECASYS KOREA).

After run the nanodrop software, chose the appropriate application nucleic acid (RNA), and 1µl of the appropriate blanking solution was added as black solution which is the same elution buffer of RNA samples (RNase –free water). After that the pedestals are cleaned and pipet 1µl of RNA sample for measurement concentration and purity for each sample.

3.2.5.4. Purity evaluation of total RNA.

A. The purity of RNA determined, by reading the absorbance in nanodrop spectrophotometer, so the RNA has its absorption maximum and the ratio 260/280 is used to assess the purity of DNA and RNA, a ratio of ~2.0 is generally accepted as “pure” for RNA, therefore selected only purity samples to treat with DNase.

B. To determine of RNA integrity in two experiment by preparing gel electrophoresis as following:

1. Agarose gel (1.5%) was prepared in 0.5 x TBE buffer (tris borate and ethylene-diamine-tetracetate buffer) and heated by using hot magnetic stirrer until thaw all crystals in agarose solution and added of 3 µl of ethidium bromide after cooling this solution.

2. The gel was poured in the tray and left until solidifying.

3. Then transferred into electrophoresis system which containing the same 0.5x TBE buffer.

4. The RNA samples were prepared by mixed 5 µl RNA samples with 1 µl of loading dye, and transferred into agarose gel wells, then running
the electrophoresis power at 100 Volt for 1 hrs. then the RNA bands are seen by ultra violet (UV) light.

3.2.5.5. DNase Treatment.

Regarding to the disadvantage of the SYBR green I, which it binds to any double-stranded DNA and produce of non-specific primer-dimers. Then treatment the extracted total RNA by DNase enzyme to remove the trace amounts of genomic DNA by using (DNase I enzyme), table (3.6), according to company instructions (BIOBASIC, USA) as the following,

1. Preparation of the tubes, latex gloves without powder, free RNase eppendorf, sterile micropipette and filter tips, all this materials apply of the U.V. sterile room below clean laminar flow.

2. Thawing the frozen RNA in ice.

3. Incubate at 37 °C for 30 minutes.

4. Add 1 µl EDTA and incubate at 65°C for 10 minutes.

5. Used the prepared RNA as a template for reverse transcriptase.

Table (3.6): DNase Treatment PreMix for preparing 10 µl from DNase-I treatment total RNA samples.

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount</th>
</tr>
</thead>
</table>

XLII
### 3.2.5.6. cDNA Synthesis.

Reverse transcription-PCR control was performed with primers for GAPDH to check the removal of all the contaminating genomic DNA. First-strand cDNA was synthesized from 1500 ng of RNA using the cDNA synthesis kit (AccuPower® RocktScript RT PreMix), following the manufacturer’s instructions. The reverse transcriptase is a modified reaction mix contains both oligo (dT), as a following steps:

1. All DNase-I treatment total RNA samples were adjusted at same concentrations that measured by DEPC water for each samples, in simple mathematic method:

\[
\text{Volume of DEPC} = \frac{(\text{required total RNA con.} \times 100) \text{ng/μl} \times \text{required volume} \times 50 \text{μl}}{\text{measured total RNA con.}}
\]

2. RNA was converted to cDNA by preparing of reverse transcription premix reaction as following (RT PreMix).

3. Add this premix into the Accu Power Rocket Script RT PreMix tubes and complemented up to 20μl by DEPC water that contains reverse transcription enzyme at lyophilized form.
<table>
<thead>
<tr>
<th>RT premix</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>15µl. (1500 ng)</td>
</tr>
<tr>
<td>Oligo dT</td>
<td>2 µl.</td>
</tr>
<tr>
<td>DEPC water</td>
<td>3 µl.</td>
</tr>
<tr>
<td>Total volume</td>
<td>20µl.</td>
</tr>
</tbody>
</table>

4. The lyophilized pellets were dissolved completely by vortex.

5. The reverse transcription reaction, converted RNA to cDNA synthesis, Performed under the following conditions, by using real time-PCR system (Excecycler 96) ® in temperature and time as blew step.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA synthesis</td>
<td>50°C</td>
<td>1 hour</td>
</tr>
<tr>
<td>Heat inactivation</td>
<td>95°C</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

6. The cDNA samples were prepared by mixed 5 µl of DNA sample with 1µl of loading dye, to determine of cDNA integrity by prepared gel electrophoresis as to above methods, then the cDNA bands are seen by U.V light, the 5µl cDNA sample with 1 µl of loading dye transferred into agarose gel wells, then running the electrophoresis power at 100 volt for one hrs., then the cDNA bands are seen by U.V light.
7. Finally, the samples were stored at -20°C until performed q (rt- PCR).

3.2.5.7. Quantitative real-time PCR.

According to method described by Wang & Hardy (2004), calculated the relative expression by q (rt-PCR) for target genes FSH-r & LH-r genes in follicular cells of DF in comparison with COF, the **ACT USING A REFERENCE GENE METHOD** can be used by normalizing gene expression of target genes (FSH-r & LH-r) with gene expression of housekeeping gene (GABDH) as a reference gene. This method used the difference between reference and target Ct values for each sample, the expression level of the reference gene are taken into account using following formula:

\[
\text{Expression value (Fold yield)} = 2^{\Delta CT \text{ (reference)}} - CT \text{ (target)}
\]

The mean of Ct numbers for target genes were normalized with reference (GABDH) gene expression for the follicular cells of DF and COF were determined by using the Microsoft excel according to Vandesompele et al.(2002), and evaluation of this results recommended by Erickson et al. (2009) depending on the following :

- Overexpression in COF > DF.
- Under expression in COF < DF.
3.2.5.7.1. Two-Step real-time PCR.

The two-step reaction, reverse to the reverse transcription amplification occur in separate tubes. (two-step rt-PCR which mean separates the reverse transcription reaction from the rt-PCR assay), two-steps protocol may be preferred when using a DNA binding dye (such as SYBR Green I) because it is easier to eliminate primer-dimmers through the manipulation of melting temperatures (Marisa & Juan, 2005).

3.2.5.7.2. Performed of q (real time-PCR).

The q(rt-PCR) was performed by using AccuPower® Greenstar™ qPCR PreMix reagent kit (Bioneer, Korea) and Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea), according to method described by Chen et al. (1999).

The SYBER Green I based q(rt-PCR) premix reagent kit is designed for PCR amplification of cDNA for target gene by using FSH-r &LH-r primer and housekeeping gene (GAPDH).

3.2.5.7.3. Experimental Design of q (real time- PCR).

For quantification of FSH-r &LH-r gene expression in dominant and cystic follicular cells, internal control gene as a housekeeping gene
(GAPDH) was used for normalization of gene expression levels, therefore, preparing three q(rt-PCR) master mixes as the following.

I)- q(rt-PCR) master mix for FSH-r target gene (forward & revers), preparing 20 µL of total volume cDNA template for these gene:

<table>
<thead>
<tr>
<th>qPCR premix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA template</td>
<td>10 µL</td>
</tr>
<tr>
<td>Primers</td>
<td></td>
</tr>
<tr>
<td>FSH-r -F</td>
<td>2 µL</td>
</tr>
<tr>
<td>FSH-r-R</td>
<td>2 µL</td>
</tr>
<tr>
<td>DEPC water</td>
<td>6 µL</td>
</tr>
<tr>
<td>Total</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

II)- q(rt-PCR) master mix for LH-r target gene (forward & revers), preparing 20 µL of total volume cDNA template for these gene:

<table>
<thead>
<tr>
<th>qPCR Premix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA template</td>
<td>10 µL</td>
</tr>
<tr>
<td>Primers</td>
<td></td>
</tr>
<tr>
<td>LH-r -F</td>
<td>2 µL</td>
</tr>
<tr>
<td>LH-r-R</td>
<td>2 µL</td>
</tr>
</tbody>
</table>
III)- q (rt-PCR) master mix for GAPDH-r gene (forward & revers), preparing 20 µL of total volume to cDNA template for these gene:

<table>
<thead>
<tr>
<th>qPCR premix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA template</td>
<td>10 µL</td>
</tr>
<tr>
<td>Primers</td>
<td></td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>2 µL</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>2 µL</td>
</tr>
<tr>
<td>DEPC water</td>
<td>6 µL</td>
</tr>
<tr>
<td>Total</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

After that, q(rt-PCR) premix were added into AccuPower GreenStar q(rt-PCR) PreMix tube, then rt-PCR tubes sealed by the optical adhesive film and mixed by vigorous vortexing for re-suspension of premix pellet. The tubes centrifuged at 3000 rpm for two minutes by using vortex/centrifuge, then start Exicycler™ 96 Real-Time Quantitative, thermal Block instrument and performed optimization for target gens to determine the performance of SYBR Green I q(rt-PCR) assay, by identifying the optimal annealing temperature for each target gene, then loaded the specific Exicycler™ 96 Program to relative quantification, according to kit instruction as the fowling:
A. Performance of Gradient Temperature Optimization.

The optimal annealing temperature can easily be assessed on Exicycler™ 96 systems which have a temperature gradient feature, according to these primers calculated annealing temperature will perform condition design (table 3.7-A; B) in one run from 63°C to 69°C for the FSH-r, LH-r and GABDH-r (forward and revers primers),

Table (3.7-A): The steps, condition and cycles by Exicycler™ 96 systems which used for gradient temperature optimization

<table>
<thead>
<tr>
<th>Step</th>
<th>Condition</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-denaturation</td>
<td>95°C for 5 min.</td>
<td>1</td>
</tr>
<tr>
<td>denaturation</td>
<td>95°C for 20 sec.</td>
<td></td>
</tr>
<tr>
<td>Gradient</td>
<td>From 63 °C, to 69°C, for 45 sec.</td>
<td>45</td>
</tr>
<tr>
<td>Melting</td>
<td>60°C to 94°C every 1°C, for 1 sec.</td>
<td>1</td>
</tr>
</tbody>
</table>

Table (3.7-B): Optimal annealing temperature degree results which obtained to each primer.

<table>
<thead>
<tr>
<th>primers</th>
<th>Result Temperature degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Primer (F,R)</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>FSH-r</td>
<td>primer (F,R)</td>
</tr>
<tr>
<td>LH-r</td>
<td>primer (F,R)</td>
</tr>
<tr>
<td>GABDH-r</td>
<td>primer (F,R)</td>
</tr>
</tbody>
</table>

**B- Performance of the Loaded the Specific Exicycler™ 96 Program to Relative Quantification, according to kit instruction.**

Both cDNA samples from dominant and cystic follicles were randomly used for PCR program which consists from initial step at 95°C for five minutes for one cycle, to activate the Taq DNA polymerase, followed by different five cycles of denaturation at 95°C for 20 seconds and a combined primer annealing/extension at the 65°C, 64°C and 67°C annealing temperature for 45 seconds for 40, 35 and 40 cycles to FSH-r, LH-r and GABDH-r successively.

**3.2.6. Statistical analysis.**

All the values are expressed as mean ± Se. data of DF and COF results were analyzed using *student t-test* and appropriate p-values of less than 0.05 were considered as statistically significant (Shiefler, 1980).
4. Results.

4.1. Samples.

The ovaries which collected from local cows divided according in to macroscopic notation in to two groups, the DF group (n=23) with diameter 19.93 ± 0.32 mm. and COF group (n=21) with diameter 37.56 ± 0.64 mm., yet there was significant difference (P≤ 0.05) in the diameter COF group in comparison with DF group, table (4.1).

4.2. Estradiol-17β and Progesterone Assay in Follicular Fluid.

The sex steroidal hormones concentration level in the FF of COF had higher E2 concentrations (865.96 ±10.64 ng/ml), than did E2 concentrations in the FF of DF (314.39 ±2.55 ng/ml), table (4.1) & figure (4.1).

The P4 concentrations levels mean in COF showed higher (84.8 ±1.35 ng/ml), compared to those in DF was (50.25 ±1.57 ng/ml), table (4.1) & figure (4.2). There was a highly significant difference (P ≤ 0.05) between two groups in E2 & P4 concentration.

Table (4.1): Differential between dominant and cystic ovarian follicles, data are presented as M± Se and t-test was used with (p≤ 0.05).
<table>
<thead>
<tr>
<th>Follicular state</th>
<th>n.</th>
<th>Follicular diameter (mm)</th>
<th>Granulosa cell layers thickness (µm)</th>
<th>Estradiol-17β conc. in F.F. (ng/ml)</th>
<th>Progesterone conc. in F.F. (ng/ml)</th>
<th>E/P &gt; 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant Follicles</td>
<td>23</td>
<td>19.93±0.32</td>
<td>18.9±0.65</td>
<td>314.39±2.55</td>
<td>50.25±1.57</td>
<td></td>
</tr>
<tr>
<td>Cystic Ovarian Follicles</td>
<td>21</td>
<td>37.56±0.64*</td>
<td>57.69±2.38*</td>
<td>865.96±10.64*</td>
<td>84.30±1.35*</td>
<td></td>
</tr>
</tbody>
</table>

(*) Significant differences.

Figure (4.1): Mean of estradiol-17β concentrations (ng/ml) in follicular fluid of dominant (n=23) and cystic ovarian follicles (n=21).

(*) Significant differences.
Figure (4.2): Mean of the progesterone concentrations (ng/ml) in follicular fluid of dominant (n=23) and cystic ovarian follicles (n=21).

(*) Significant differences.

4.3. Histological Study.

The thickness of the granulosa cell layers determined by measuring the image on the light microscope (ocular lens and stage), that the granulosa cell layer was present in DF and COF (figures 4.3 & 4.4), but it's thinner in DF (18.9±0.65 µm), while high thickness in COF (57.69 ± 2.38 µm), figure (4.5) and table (4.1), it will be seen that the cysts group contain highly significant thickness (P ≤ 0.05) than granulose cell layer of DF.
Figure (4.3): Sections of dominant follicular wall, (S) follicular space; (G) granulosa cell layer; (T) theca cell layer, (H&E, 10x & 40x).
Figure (4.4): Sections of cystic follicular wall, (S) follicular space; (G) granulosa cell layer; (T) theca cell layer, (H&E, 10x & 40x).

Figure (4.5): Mean of the granulosa cells thickness (µm.) in dominant (n=23) and cystic ovarian follicles (n=21).

(*) Significant differences.

4.4. Molecular Analysis.

4.4.1. Quantification of total RNA.

The extraction of total RNA from follicular cells pellet which isolated in two experiments by using Reagent (Accuzol Usere manual, BIONEER-Korea) according to the manufacturer’s instructions, then RNA concentration measured by Nano drop spectrophotometer.

The value of total RNA concentration in first method was very scanty (21.276 ± 3.06 ng /µl) in follicular cells mashed of DF, and 25.12 ±2.3 ng /µl in COF as a table (4.2), therefore leave these results and depend on...
second method, it has been appeared suitable high total RNA concentration in this method are 94.374 ± 3.07 ng/µl in follicular cells mashed of DF, while in COF are 95.64 ± 2.98, the statistical analysis refer to highly significant difference between first & second methods \( (P \leq 0.05) \). The second method has greater mean of total RNA concentration value in DF & COF groups, in this study will be depend on this results in flowing steps, table (4.2).

Table (4.2): The total RNA concentration (ng/µl) in follicular cells mashed of dominant and cystic ovarian follicle which isolated by first and second methods, data are presented as M± Se and \( t \)-test was used with \( (p \leq 0.05) \).

<table>
<thead>
<tr>
<th>Follicular state</th>
<th>n.</th>
<th>Mean of total RNA concentration in follicular cells mashed (ng/µl).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>First method</strong></td>
</tr>
<tr>
<td>Dominant Follicles</td>
<td>23</td>
<td>21.276 ± 3.06</td>
</tr>
<tr>
<td>Cystic Ovarian Follicles</td>
<td>21</td>
<td>25.12 ± 2.3</td>
</tr>
</tbody>
</table>

(*) Significant differences.
4.4.2. Purity evaluation of total RNA.

Total RNA concentrations and purity were assessed using nanodrop spectrophotometer in absorbance readings ratio (260/280 nm). All samples that used in the second experiment gave high concentrations of total RNA, and good purity ratio (1.7-1.9) of total RNA.

The purity of total RNA samples, also assessed by using agarose gel electrophoresis of follicular cell of DF & COF, each sample was presented two bands (18S rRNA and 28S rRNA) as well as smear of mRNA. In first experiment, the bands of RNA (low concentration) were degradation and absence in many samples, yet these bands in second experiment were good and clear with presented two bands (18S rRNA and 28S rRNA), figure (4.6)
Figure (4.6): Agarose gel electrophoresis of RNA (28S-rRNA & 18S-rRNA) in follicular cell that obtained from secondary experimental. dominant follicles (1, 2, 3, 4) and cystic follicles (5, 6, 7, 8).

4.4.3. cDNA Synthesis.

All the total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided from BIONEER company, Korea in reverse transcription reaction, for converted RNA to cDNA synthesis by using rt-PCR system (Excecycler 96) ®. In temperature and time as in chapter three, this products reader by electrophoresis, then the cDNA bands were seen by U.V light, as figure (4.7).

Figure (4.7): Agarose gel electrophoresis analysis of cDNA in follicular cells samples of dominant follicles (1,2,3,4,5,6) and cystic follicles (7,8,9,10,11).
4.5. Quantitative real time-PCR.

Data analysis of SYBR green I based rt-PCR assay were divided into primer efficiency estimation and relative quantification of FSH-r& LH-r genes expression level which normalized by housekeeping gene expression (GAPDH).

4.5.1. Gradient Temperature Optimization.

The data result, threshold cycle numbers (Ct) were calculated from amplification plot of rt-PCR detection system, during exponential phase of fluorescent signals of SYBR green I primer of FSH-r & LH-r genes, that react with complementary DNA (cDNA) of follicular cells mRNA, where, the amount of PCR product (DNA copy numbers) in master mix reaction is approximately doubles in each PCR cycle to check the annealing temperature for each primers (FSH-r & LH-r) and (GABDH) forward and revers were (65°C, 64°C and 67°C) for this primers successively by taken the lower Ct value in amplification plot results.

4.5.2. Relative Quantification of Target Gene Expression.

To calculate the relative expression of target gene in follicular cells of the DF & COF, the $2^{ΔCt}$ using a Reference Gene Method used by normalizing target genes expression of FSH-r & LH-r genes with expression of housekeeping or reference gene (GAPDH). This method used the difference between reference and target Ct values for each sample, the expression level of the reference gene is taken into account.
Of the Ct number of target genes normalized to that of reference gene in all test (COF group) and calibrator (DF group). So, the ΔCt of test group and the ΔCt of calibrator group are normalized too, finally the expression ratio (fold change) was calculated by \(2^{\Delta Ct}\) method, table (4.3) & figures (4.8).

Calculate the relative expression, simply normalize FSH-r & LH-r gene expression for each sample by following formula-1.

\[
\text{Expression (gene yields)} = 2^{\Delta Ct} = 2^{(\text{Ct (GAPDH)} - \text{Ct (target)})}
\]

Finally, the expression ratio is calculated by formula-2.

\[
\text{Normal expression} = \frac{\text{Normal}}{\text{Normal}} = 1
\]

\[
\text{Test expression} = \frac{\text{Test}}{\text{Normal}} = \frac{\text{Test}}{1} = >1.5 \text{ high expression (up-regulation)}
\]

\[
\text{or} <1.5 \text{ Low expression (down-regulation)}
\]

Table (4.3): The mean of Ct values and expression value of the FSH-r gene & LH-r genes in the follicular cells of dominant and cystic follicles.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mean of CT values</th>
<th>ΔCT</th>
<th>((2^{\Delta CT})) Expression value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABDH-r</td>
<td>Target gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dominant Samples (n=14)</td>
<td>Cystic Samples (n=14)</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.1680</td>
<td>20.8869</td>
<td></td>
</tr>
<tr>
<td><strong>FSH-r</strong></td>
<td>21.2844</td>
<td>19.8691</td>
<td></td>
</tr>
<tr>
<td><strong>LH-r</strong></td>
<td>17.2774</td>
<td>27.5590</td>
<td></td>
</tr>
<tr>
<td><strong>Ct value</strong></td>
<td>-1.1164</td>
<td>1.0177</td>
<td></td>
</tr>
<tr>
<td><strong>Ct value</strong></td>
<td>0.46124</td>
<td>2.0246</td>
<td></td>
</tr>
</tbody>
</table>

**A-The Ct value GABDH-r.**

The Ct value GABDH-r = 20.1680 in DF, and 20.8869 in COF, figure (4.9).

**B-Relative Quantification of FSH-r gene Expression.**

FSH-r mRNA relative expression down-regulated (0.46124) in DF, and up-regulated (2.0246) in COF (figure-4.10), therefore the expression ratio of FSH-r mRNA are:
C-Relative Quantification of LH-r gene Expression.

LH-r mRNA relative expression up-regulated (7.4127) in DF, and down-regulated (0.0098) in COF (figure -4.11), therefore the expression ratio of LH-r mRNA are:

Normal expression ratio = 7.4127/7.4127=1
Test expression ratio = 0.0098/7.4127= 0.0013 yield of this gene in follicular cells of COF (low expression or down-regulation)
Figure (4.8): Fold change of mRNA transcript levels of the FSH-r gene & LH-r genes in the follicular cells of dominant and cystic follicles.

Figure (4.9): Real Time PCR Amplification plot for GABH-r gene in dominant & cystic follicular cells by Excecycler96® system.
Figure (4.10): Real Time PCR Amplification plot for FSH-r gene in dominant & cystic follicular cells by (Execycler 96) ® system.
5. Discussion.

It’s a first thesis to depict comparison analysis of gene expression of the FSH-r & LH-r gene in ovaries of local Iraqi breed by using the q (rt-PCR) in order to identify new molecules useful for discrimination of bovine ovaries with and without cystic follicles.

5.1. Dominant and Cystic Follicular Diameter.

This study demonstrated that the high differential (p≤ 0.05) significant of follicular diameter size of COF (37.56 ± 0.64 mm) compared with DF (19.93 ± 0.32 mm), table (4.1). These result findings consider characteristic of cystic follicles by the presence of a high volume of follicular fluid, and agreements with more than one like (Vanholder et al., 2006 and Youngquist & Threlfall, 2007).

5.2. Estradiol & Progesterone Assay in Follicular Fluid.
The follicular fluid of COF had higher E2 concentrations (865.96 ±10.64 ng/ml) than follicular fluid of the DF (314.39 ±2.55 ng/ml), yet the concentration of P4 was depressed (50.25 ±1.57 ng/ml) in DF, in compared with the concentration of COF (84.308 ±1.35 ng/ml), (table 4.1).

The E2/P4 ratio was greater than one that’s indication to the DF came from ovaries in the follicular phase of the estrus cycle and hormonally classified as healthy (estrogen active) according to Mihm et al. (2006) also the Boryczko et al. (1995) were classified the COF to estrogen-active cysts because this ratio greater than one (E/P > 1) and concentration of P4 less than hundred (P < 100 ng/ml) in FF, yet this hormonal results was complete agreement with many previous studies (Fortune et al., 2001; Calder et al., 2001 and Beg & Ginther, 2006). The granulosa cells are the main site of production E2, therefore the high concentrations of E2 can result in genetic alterations, yet the elimination of these cells will reduce the E2 concentration (Isobe & Yoshimura, 2007), these findings support diagnosis our finding in present study.

5.3. Histological study of Follicular Wall in Dominant and Cystic Follicles.

The histological result of the dominant and cystic follicle wall were found that the higher thickness of the granulosa cell layer in COF than DF, The width of the granulosa cell layer was smaller 18.9±0.65 µm (P ≤ 0.05) in DF as a compared in COF 57.69±2.38 µm, table (4.1). These findings were in agreement with Rodgers et al. (1999); Isobe et al. (2008) and Grado-Ahuir et al. (2009). This results may be indicated that
granulosa and theca cells of COF is related with delays follicular regression, on the other hand the granulose cells are the main site of production of estradiol, and the follicular fluids of COF distinguished with high concentration of E2 and a low concentration of P4. These findings support our findings in diagnosis of COF in present study.

5.4. Relative Quantification of FSH-r & LH-r Genes Expression in Dominant and Cystic Follicles.

The genomic relationship between FSH-r and LH-r, when examined together with their similar expression profiles and functions in gonadotropin signaling raise the intriguing possibility that the function of one or both genes depends on their relative positions (Hunzicker-dunn & Mayo, 2006). In this study, used a q(rt-PCR) assay to measure relative quantification of mRNA transcript levels (gene expression) for FSH-r & LH-r genes, with housekeeping gene (GABDH), for explanations pathogenesis of the COF and regarding synthesis of these receptors in the follicular cells of DF and COF.

5.4.1. Expression of FSH-r Genes.

In cows with normal estrous cycles, the FSH-r is localized in granulosa cells of follicles of all diameters, and the mRNA was expressed in both theca and granulosa in DF & COF (Xu et al., 1995 and Luo et al., 2005).

The results of the present study confirm and extend the notion that there is a relative mRNA expression for the FSH-r genes was high-regulated in follicular cells of COF alternative for mRNA expression of
this genes in follicular cells of DF, table (4.3) & figure (4.8). This a high regulated result agree with previous studies (Themmen, 1991; Ginther et al., 2003a and Mihm et al., 2006) whose indicating the increase of FSH-r on the granulosa cells of dominant follicle. Because of the ovarian follicles development depends up on FSH action therefore any decline in mRNA expression for this receptors lead to disturbance in growth and function of dominant follicles, furthermore the mutation of the FSH-r genes lead to lack of FSH function which are affected ovarian follicular development, that lead to ovulation failure and infertility, but studies of Dunkel et al. (1994); Xu et al. (1995) and Braw-Tal1 & Roth (2005) observed that the level of expression of FSH-r mRNA decreased coincident with growth of the dominant non ovulatory and ovulatory follicles.

Ginther et al. (2003a) was explicit that the steroidal hormones concentration and FSH itself, earlier reports of both up- and down-regulation, the up-regulation refers to increase in the number of receptor site, yet the E2 up-regulates FSH-r sites, whereas the FSH stimulates granulosa cells to produce E2. This synergistic activity of estrogen and FSH allows follicular growth and development in the ovary (Themmen, 1991 and Minegishi et al., 2000).

In adult animals, LH receptor mRNA levels change dramatically during the estrous cycle, particularly after the pre-ovulatory LH surge, and after the follicle selection which is associated with an increase in mRNA for FSH-r in granulosa cells of DF and a decrease in mRNA for LH receptor in theca cells of subordinate follicles. (Evans & Fortune, 1997), but Kawate et al. (1990) observed that the FSH-r & LH-r numbers in granulosa cells of cysts are decreased when compared to normal
follicles. The effects of increased estradiol production and FSH action on the granulosa cells of dominant follicles may stimulate the expression of LH receptors in granulosa cells, to respond to the pre-ovulatory LH surge, and because of the estrogen and FSH are required for stimulating the expression of LH-r or its mRNA in granulosa cells according to Fortune et al. (2001) and Beg & Ginther (2006).

The growth of dominant follicles during the low FSH of ovarian follicular waves is associated with down regulation expression of the FSH-r genes but the LH-r genes involved with proliferation of theca and (or) granulosa cells. The current study used a q(rt-PCR) to identify, what gene /or genes that potentially regulate proliferation and survival in follicular cells and in expression of receptor mRNA as a key for hormones (FSH&LH) that regulate antral follicular growth.

5.4.2. Expression of LH-r Genes.

The results of the present study confirms and extends the notion that there is a relative mRNA expression for the LH-r genes was highly down-regulated in follicular cells of COF alternative for mRNA expression of this genes in follicular cells of dominant follicles, table (4.3) & figure (4.8). which are high-regulated, these results agree with the observation of Cupp et al.(1993); Ascoli et al.(2002) and Nogueira et al. (2007) whose referring to increase of the LH-r in DF that remain estrogenic for prolonged period when exposed to low P4/high LH pulse frequency, furthermore this receptor concentrations in granulosa cells increased with follicle diameter (Camp et al.,1991 and Peng et al., 1991).The study of Kawate et al.(1990) and Robert et al.(2003) were showed that the
increased of the number of LH-r protein in follicular cells, rapidly in the latter stage of antral follicular development furthermore the expression of LH-r protein increases as the follicles mature to become graafian follicles, therefore the granulosa cells of preovulatory follicles which have insufficient expression of LH-r as a result the ovaries were unable to fully respond to LH pulse, leading to a reduced rate of follicle rupture.

The LH concentrations were increased in cystic animals according of Hamilton et al. (1995), but Calder et al. (2001) observed lower or even absent LH-r mRNA transcripts in theca and granulosa cells of COF, on the other hand, Odore et al. (1999) observed similar receptor which have a high concentrations in COF and DF, or lack of LH receptors in this cells (Johnson, et al., 1997 and Robker, et al., 2000). Furthermore Bao et al. (1997); Luo et al. (2005); Mihm et al. (2006) and Nogueira et al. (2007) reported that the LH-receptor mRNA was about eight times higher in the dominant follicle, yet when compartment this results (highly down-regulated of the LH-r genes in COF) with previous studies which clearly established that the LH-r protein expression in the follicular cells was down-regulation of LH-r protein expression for prevent the ovary from repeated stimulation to pre-ovulatory surge of LH, which causes transient desensitization of LH response. This alteration may be the cause of COF (Kawate et al., 1990 and Robker et al., 2000) which might be due to fact that the LH-r protein number reduced in follicular cells of COF, also Cook et al. (1991) and Hamilton et al. (1995) showed that the LH-r mRNA expression was numerically but not significantly increased in DF as compared with COF. The changes in the expression of the various transcripts during up- and down-regulation of FSH and LH receptors expression follow a similar pattern under several experimental models.
This results of the present study may also explained the reduction rate of LH-r synthesis is not due to decrease transcription but rather to reduced LH-r mRNA half-life, these findings were disagreed with Cupp et al. (1993) and Calder et al. (2001) who found that there are no significant differences in FSH-r & LH-r mRNA were observed between the dominant follicles and young cysts, but the LH-r mRNA expression increased in cystic cow, due to estrogen effect for prolonged periods when exposed to low P4/high LH pulse frequency as compared with dominant follicles. This high expression of granulosa LH-r mRNA may contribute to increase follicular steroidogenesis, while Ireland & Roche, (1983) and Lu et al. (1993) claimed that the reasons for this discrepancy are not known and could be related to differences in tissue preparation and identification of healthy follicles, also the changes in the steady-state could result from either a decreased rate of receptors genes transcription and/or an increase in the rate of receptor mRNA degradation.

6. Conclusions and Recommendations


1- The transcription levels of LH-r gene altered down regulation of LH-r gene expression in cells of the COF, may be assistance to understand the etiology and pathology of this case (disease).
2- The up-regulation of the LH-r gene in follicular cells of DF explained the important role of the LH in ovulation mechanism and increasing follicular ovulation chance.
3- The down-regulation of the FSH-r gene in follicular cells of DF lead to decrease in FSH receptors numbers on this cells and lower sensitization to FSH, that causes un increase of follicular cells responsible to effect of LH pulsation.
4. The presence of enough FSH receptors on cystic follicles because of the up-regulation of FSH-r gene assistance of its persistence follicles to growth continues due to FSH effect.

6.2. Recommendations.

1. Investigation the overlapping role of the alternative FSH-r & LH-r genes expression in the follicular cells of DF & COF for local cow with availability of the case history.

2. Using real-time PCR for quantitative mRNA transcription levels to other hormonal receptor gene which associated with fertility in local breeding cow.

3. Developing a national animal gene information system in Iraq which supports, the development of breeding cows, to explain pathogeneses and etiology of infertility diseases.

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