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Estimation of Kisspeptin and Its Relationship with Some Hormonal and Biochemical Parameters in Different Stages of The women's menstrual Cycles in Maysan Province

A Thesis

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by

Noora Kareem Abdul Hasan

B.Sc. Biology (2006)

Supervised by

Prof. Dr. Ahmed Aboud Khalifa

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بِسْمِ اللهِ الرَّحْمَٰنِ الرَّحِيمِ

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We certify that this thesis entitled **"Estimation of kisspeptin and its relationship with some hormonal and biochemical parameters in different stages of the women's menstrual cycles in maysan province "**has been prepared under our supervision at the college of science, university of misan; as a partial fulfillment of the requirements for the degree of master of biology.

Signature

Prof. Dr. Ahmed Aboud Khalifa

Department of Biology

College of Science / Misan /University

Date: ... /… /….

Recommendation of Head of Biology Department

In view the available recommendations; I forward this thesis debate by examining committee.

Signature

Assist. Prof. Dr. Maytham A. Dragh

Head of Department of Biology

College of Science / Misan/University

Date: .../ … / …...

Dedication

To the source of tenderness and motherhood... my beloved mother...

To the corner of safety and the comfort of warmth... my dear father...

To the companion of the soul and the beat of the heart, to the one who overcame the difficulties and melted them in my way... my beloved husband...

To the pleasures of my liver and the flower of my life, my children Sajad, Zahraa, Wadek, Ali, Ahmed…

To the beacons of my soul, companions of the paths of my life, my support, my brothers.

To my beloved and the loss of my heart (God have mercy on her soul) Dima...

To everyone who taught me a letter since childhood and did not skimp on advice and guidance...

To every science lover...

I dedicate this research

Noora Kareem

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Summary

Summary

The present study aimed to estimate the kisspeptin hormone and its relation with some hormonal and biochemical parameters in different phases of the women's menstrual cycle in Maysan province. The sample included thirty healthy women (aged 20-45 years) whom visited the child and birth governmental hospital and some clinical centers divided into two groups (15 women /group) according to their ages as following:

- The first group: 20-25 years.
- The second group: 40-45 years.

Blood samples were drawn in eighth, sixteenth and twenty fourth days of the menstrual cycle.

The present results revealed that:

1- Kisspeptin levels increased significantly ($p \leq 0.05$) in the 16^{th} day in comparison with the 24^{th} day and non-significantly with the 8^{th} day for the first and second groups, respectively. In addition, its increased significantly ($p \leq 0.05$) (except 24^{th} day) at the second group in comparison with the first group for the similar days.

2- Follicular stimulating hormone (FSH) levels increased significantly ($p \leq 0.05$) in the 8^{th} day (excepted 16^{th} day in the first group) in comparison with the 16^{th} day in the second group and the 24^{th} day of both groups. In addition, its increased significantly ($p \leq 0.05$) at the second group in comparison with the first group for the similar days.

3- Luteinizing hormone (LH) levels increased significantly ($p \leq 0.05$) in the 16^{th} day in comparison with the 8^{th} and 24^{th} days of both groups. In addition, its increased non-significantly at the second group in comparison with the first group for the similar days.

4- Estradiol levels increased non-significantly in the 8th day in comparison with 16th day and significantly ($p \leq 0.05$) in comparison with the 24th day of both groups. In addition, it's decreased non-significantly at the second group in comparison with the first group for the similar days.

5- Progesterone levels increased significantly ($p \leq 0.05$) in the 24th day in comparison with the 8th and 16th days of both groups. In addition, its decreased non-significantly at the second group in comparison with the first group for the similar days.

6- Prolactin levels increased significantly ($p \leq 0.05$) in the 16th day in comparison with the 8th day and non-significantly with the 24th day of both groups. In addition, its increased significantly ($p \leq 0.05$) at the second group in comparison with the first group for similar days.

7- Hydrogen peroxide ($H_2O_{2)}$ levels increased non-significantly in the 16th day in comparison with the 8th day and significantly ($p \leq 0.05$) with the 24$_{th}$ day of both groups. In addition, its increased significantly ($p \leq 0.05$) at the second group in comparison with the first group for the similar days.

8- Catalase (CAT) levels increased non-significantly in the 8th day in comparison with the 16th day and significantly ($p \leq 0.05$) with the 24th day. In addition, its increased significantly ($p \leq 0.05$) at the second group in comparison with the first group for the similar days.

9- Lecithin-cholesterol acyltransferase (LCAT) levels increased non-significantly in the 8th day in comparison with the 16th day and significantly ($p \leq 0.05$) with the 24th day. In addition, its increased non-significantly at the second group in comparison with the first group for the similar days.

10- Granulocyte colony-stimulating factor (G-CSF) levels increased non-significantly in the 16th day in comparison with the 8th day and significantly ($p \leq 0.05$) with the 24th day. In addition, its increased significantly ($p \leq 0.05$) at the second group in comparison with the first group for the similar days.

11- Total white blood cells (WBC) increased non-significantly in the 16th day in comparison with the 8th day and significantly ($p \leq 0.05$) with the 24th day. In addition, its increased significantly ($p \leq 0.05$) at the second group in comparison with the first group for the similar days.

12- Total cholesterol (TC) levels increased non-significantly in the 8th day in comparison with the 16th day and increased significantly ($p \leq 0.05$) with the 24th day. In addition, its increased non-significantly at the second group in comparison with the first group for the similar days.

13- Triglyceride (TG) levels increased non-significantly in the 8th day in comparison with the 16th day and increased significantly ($p \leq 0.05$) with the 24th day. In addition, its increased non-significantly at the second group in comparison with the first group for the similar days.

14- High density lipoprotein (HDL) levels increased significantly ($p \leq 0.05$) in the 16th day in comparison with the 8th and 24th days. In addition, its decreased significantly ($p \leq 0.05$) at the second group in comparison with the first group for the similar days.

15- Low density lipoprotein (LDL) levels increased significantly($p \leq 0.05$) in the 8th day in comparison with the 16th and 24th days. In addition, its increased non-significantly at the second group in comparison with the first group for the similar days.

16- Very low-density lipoprotein (VLDL) levels increased non-significantly in the 16^{th} day in comparison with the 8^{th} day and significantly ($p \leq 0.05$) with the 24^{th} day. In addition, its increased non-significantly at the second group in comparison with the first group for the similar days.

The physiological impacts of these results discussed according to the influence of menstrual cycle's phases and progressive age followed with a low ovarian efficiency and consequently hormonal, oxidantal and biochemical alterations.

And concluded that kisspeptin mediates and controls a network of hormones , oxidants / antioxidant, proinflammatory marker and lipides that mange and regulate the ovulation process and all its related aspects during the menstrual cycle.

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List of Abbreviations

ABBREVIATIONS	
ABCA1	ATP binding cassette transporter-A1
AKT	Serine/threonine kinase
APO A1	Apolipoprotein-A1
ARC	Arcuate nucleus
ARRβ2	β-arrestin 2
AVPV	Anteroventral periventricular nucleus
C7αOH	Cholesterol 7α-hydroxylase
CAT	Catalase
ERα	Estrogen receptor α
FGF	Fibroblast growth factor
FOXO1	Forkhead box protein O1
FSH	Follicle- stimulating hormone
FSHRs	FSH receptor
G-CSF	Granulocyte colony - stimulating factor
GM-CSF	Granulocyte-macrophage colony factor
GnRH	Gonadotropin- releasing hormone
GPCRs	G-protein coupled receptors
GPR54	Kiss 1-drived peptide receptor
GPX	Glutathione peroxidases
GRX	Glutathione reductase

GSH	Glutathione
H_2O_2	Hydrogen peroxide
HDL	High density lipoproteins
HL	Hepatic lipase enzyme
HMG-COA	Hydroxy methylglutaryl-co A
HMGCR	Hydroxy methylglutaryl co enzyme A reductase
HPG	Hypothalamic-pitutary-gonadal
IFN-y	Interferon-y
IL8	Interleukin 8
IP-10	IFN-y induced protein 10
KP 10	Kisspeptin10
KP 13	Kisspeptin13
KP 14	Kisspeptin 14
KISS 1	Kisspeptin gene
Kiss 1R	Kisspeptin receptor
KNDy	Kisspeptin, Neurokinin and Dynorphin.
KP 54	Kisspeptin 54
LCAT	Lecithin - cholesterol acyltransferase
LDL	Low density lipoprotein
LH	Luteinizing hormone
LPL	Lipoprotein lipase
MAD	Malondialdehyde
MCP1	Monocyte chemoattractant protein 1`
MIP-1α	Macrophage inflammatory protein 1alpha
MIP-1β	Macrophage inflammatory protein 1beta
mRNA	Messenger RNA

OS	Oxidative stress
P38 MAPK	P38 mitogen-activated protein kinases
P450	Cytochrome P
PDGF	Platelet derived growth factor
PGF2α	Prostaglandin F2α
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone receptor
RANTCES, EOTAXIN	Regulated on activation, normal T cell expressed and secreted
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SR-B1	Scavenger receptor class B type 1
SREBP2	Sterol regulatory element-binding protein
TAC	Total antioxidant capacity
TC	Total cholesterol
TG	Triglyceride
TIDA	Tuberoinfundibular dopamine neuron
TNFα	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor
VLDL	Very low - density lipoprotein
WBC	White blood cells

Chapter One

Introduction

Introduction

Female's reproductive system exhibits regular cyclic alterations that, could be seen as periodic preparation for conception and fertilization, named a menstrual cycle that have a most noticeable feature (the sporadic vaginal bleeding), whereas, results by the shedding of uterine mucosa (menstruation) (Coast *et al*.,2019; Pan and Li, 2019).

Normal menstrual cycle in average last about twenty eight days from beginning till the next, consists of follicular phase and luteal phase, mediates by ovulatory phase, these events occurred as a result of the pulsating release of hypothalamic gonadotropin-releasing hormone (GnRH) that, in turn stimulates the anterior pituitary gland to secret follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which stimulate ovarian estrogen and progesterone secretion (Itriyeva , 2022).

Many studies showed that more hypothalamic hormones contributed in GnRH release and had been played an important role in female reproduction such as neurokinin B, dynorphin and kisspeptin (Herbison, 2016; Harter *et al*., 2018).

Kisspeptin is an essential element in creating LH surge and ovulation and its expression increments just before ovulation and LH surge (Nejad *et al*.,2017; Trevisan *et al*.,2018). Kisspeptin neurons are primarily located in the preoptic area and hypothalamic arcuate nucleus and function as upstream regulators of GnRH neurons (Katulski *et al*.,2018). Furthermore, kisspeptin directly stimulates GnRH secretion via Kiss1-derived peptide receptor (GPR54) expressed in GnRH neurons in rodents (Uenoyama *et al*., 2021).

On the other hand, the menstrual cycle accompanied with occurrence of some reactive oxygen species (ROS) such as malondialdehyde (MDA), 8-isoprostane and hydrogen peroxide (H_2O_2) (Całyniuk *et al*., 2016; Prasad *et al*., 2016; Graille *et al*., 2020). H_2O_2 is one of these compounds that interferes with basal steroid production and inactivate the gonadotropin–receptor complex, these anti-steroidogenic action of H_2O_2 raises the possibility of a modulatory role of H_2O_2 in human luteal steroidogenesis (Agarwal *et al.*,2005; Vu and Acosta, 2018).

On the other wise, the detrimental effects of the ROS be fighted and combatted by some anti-oxidantal factors such as superoxide dismutase (SOD), glutathione peroxidases (GPX), catalase (CAT) and lecithin-cholesterol acyltransferase (LCAT) (Meca *et al*., 2021; Yang *et al*., 2022).

CAT is one of the enzymes that have the ability to convert millions of hydrogen peroxide molecules to water and oxygen each second (Ighodaro and Akinloye,2018) and CAT may represents a dominant antioxidant defense in the initial stages of folliculogenesis (Gupta *et al*., 2011). LCAT plays an important role in reverse cholesterol transport and follicular synthesis of estrogen (Cigliano *et al*., 2002) and contributed in steroidogenesis via its cholesterol attachment with high-density lipoproteins HDL in ovaries (Fujimoto *et al*., 2010; Chang *et al*., 2017).

On the other hand, numerous studies showed that inflammation processes are involved in ovulation and play a key role in ovarian follicular dynamics (Rostamtabar *et al*.,2021). In addition, many cytokines were detected in follicular fluid (such as granulocyte colony-stimulating factor G-CSF), they influence the development and maturation of the follicle, ovulation, and corpus luteum formation (Yanagi *et al*.,2002; Adamczak *et al*., 2021). G-CSF is a multifunctional cytokine that is well recognized for increasing neutrophil

activation and production during hematopoiesis , as well as for playing a crucial part in immune and inflammatory responses (Basu *et al.*, 2002; Bendall and Bradstock, 2014) , moreover , the intrafollicular G-CSF production by granulosa cells and its higher concentration in follicular fluid than in serum (Salmassi *et al*.,2004) , furthermore , Noel and his colleagues 2020 demonstrated that G-CSF has an ability to recruit and activate neutrophils , might play a role in the mechanism of oocyte maturation and subsequent ovulation .

Furthermore, Yanagi and his colleagues 2002 concluded that G-CSF produced in the human follicle shortly before the ovulatory phase and might play an important role in the mechanism of ovulation.

Moreover, lipoprotein cholesterol levels have been changed during menstrual cycle in response to varying reproductive hormones levels, triglyceride (TG) and low-density lipoprotein (LDL) tend to be highest during the follicular phase and to decline during the luteal phase, high-density lipoprotein (HDL) is most often highest during the late follicular and periovulatory phases (Mumford *et al*.,2011).

On the other hand, studies have been shown that many changes and deterioration in kisspeptin levels, some reproductive hormones, reactive oxygen species (ROS), antioxidants and inflammatory parameters in menstruating women with advancing age, particularly, years before the disappearance of menstrual cycle (premenopause).

The increase of kisspeptin expression (Drobintseva *et al.,*2021), estrogens, progesterone decrease and the rise of FSH and LH (Mersereau *et al*., 2008) had been detected in menstruation women during premenopause, moreover, with aging process there are increased in oxidative stress and an increase the release

of H_2O_2 that related with decreased estradiol in menstruated women (Moreau *et al.*,2020). Furthermore, the concentrations of some antioxidative biomarkers such as CAT and LCAT decreased remarkedly in menstruated perimenopausal women when compared with younger women (Zern *et al*., 2005; Siregar *et al*., 2015). Moreover, the inflammatory cytokines G-CSF increased in menstruated women aged 36-46 years in compared with younger menstruated women (Yanagi *et al*.,2002), in addition, Abbak and his colleagues 2019 mentioned that both LDL cholesterol and triglycerides increased while HDL cholesterol decreased in menstruated premenopausal women.

Aims of the study:

In view of these controversy, this study is an attempt to shed some light about the role of the kisspeptin and its relationship with some hormonal and biochemical parameters in different phases of the young and old women's menstrual cycles in Maysan province.

This research was conducted to investigate the following parameters:

1. Kisspeptin.
2. FSH.
3. LH.
4. Estradiol.
5. Progesterone.
6. Prolactin.
7. H_2O_2.
8. CAT.
9. LCAT.
10. G-CSF.
11. WBC count.
12. Lipid profile

Chapter Two
Literature Review

2.1 Kisspeptin…An overview

Kisspeptin was firstly recognized in 1996 as a human metastasis suppressor of malignant melanoma (Lee *et al*.,1996). Its encoded by the KISS1 gene (West *et al*.,1998) , generates 145-amino acid prepro-kisspeptin that is unstable , physiologically inactive and cleavages inside the cell for four physiologically active peptides : kisspeptin-54 (KP54) , kisspeptin-14 (KP14) , kisspeptin-13 (KP13) and kisspeptin-10 (KP10) (Roseweir and Millar,2009) , KP54 has been considered as the main product of the KISS1 gene in human and it can be further cleaved into KP14 , KP13 and KP10 (Hu *et al*.,2022), moreover , kisspeptin receptor (KISS1R) belongs to the family of the seven transmembrane G-protein-coupled receptor , which binds extracellular substances and transmits signals to an intracellular molecule (Lee *et al*., 1999) and considered one of the most important G-protein coupled receptors (GPCRs) in the neuroendocrine control of reproductive function and its ligand kisspeptin has a significant effect on the hypothalamus (Shahab *et al*.,2005).

Furthermore, kisspeptins are a family of neuropeptides with regulatory functions related to puberty, fertility and reproduction (Ahart *et al*.,2021), kisspeptin is synthesized in two major sections of the hypothalamus the arcuate nucleus and the anterior ventral periventricular nucleus (Marraudino *et al*.,2018) , whereas , they have an essential role in the central regulation of the hypothalamic-pituitary-genital axis, furthermore, it had been discovered that hypothalamic networks which control GnRH secretion and steroid feedbacks , the neuropeptides in this network include (kisspeptin, Neurokinin and Dynorphin) (KNDy) , via gap junctions the neuron-neuron and neuron-glia communicate and contribute with the synchronized activities among KNDy neurons (Ikegami *et al*., 2017), in addition, kisspeptin and his receptor (KISS1R) are expressed in the mammalian ovary and kisspeptin signals may have a putative role in the direct regulation of ovarian function, including

follicle development, oocyte maturation, steroidogenesis and ovulation (Hu *et al.*,2018).

Moreover, kisspeptin besides its pivotal role in LH control, it's also influencing prolactin secretion, its regulation of prolactin secretion may involve at the concurrent reduction in dopamine activity (the main hypothalamic hormone regulating prolactin secretion), which probably facilitates prolactin secretory response (Aquino *et al*.,2019).

Furthermore, kisspeptin have a potential role in the preparation of endometrial tissue for implantation due to kisspeptin is expressed in the endometrial epithelial and stromal cells, but not in the myometrium (in human uterus) (Cao *et al*., 2019), thereby, kisspeptin may also act as a mediator that facilitates implantation of the growing embryo to the uterine wall (Ahart *et al*.,2021).

In addition, kisspeptin have role as an anti-oxidant effect, whereas, kisspeptin overexpression alleviated the accumulation of ROS and enhanced the levels of the antioxidants GSH, SOD, and CAT through activation of the PI3K/AKT signaling pathway, indicating that kisspeptin protects ovarian granulosa cells from oxidative stress (Huang *et al*.,2021; Sun *et al*.,2023).

On the other hand, many evidences indicated to the role of kisspeptin in regulating metabolic processes, whereas, kisspeptin exerts its metabolic effects indirectly via gonadal hormones and/or directly via the kisspeptin receptor in the brain, pancreas and brown adipose tissue, kisspeptin receptors knockout studies indicate that kisspeptin may play sexually dimorphic roles in the physiological regulation of energy expenditure, food intake and body weight, moreover, in vivo and in vitro studies demonstrated that high kisspeptin concentrations enhanced of glucose-stimulated insulin secretion (Izzi-Engbeaya and Dhillo ,2022).

2.2 Kisspeptin and Menstrual Cycle

Menstrual cycle represents a coordinated serial event, including (follicular phase, ovulatory and luteal phase), repeated month by month, at regular intervals, in which the hypothalamus participates secreting GnRH, along with the anterior pituitary gland secreting FSH and LH, mediated by kisspeptin (Leonardi *et al*.,2020).

Kisspeptin neurons are primarily located in the preoptic area and hypothalamic arcuate nucleus and function as upstream regulators of GnRH neurons (Katulski *et al*.,2018), both of these anatomic locations, serum estrogen and progesterone concentrations have been shown to regulate kisspeptin-mediated GnRH secretion (Meczekalski *et al*.,2016).

Moreover, a study by Meczekalski and his collagenous 2016 demonstrated that each kisspeptin pulse is accompanied by a pituitary LH pulse in response to a hypothalamic GnRH pulse, in addition, the blockade of the kisspeptin receptor (GPR54) resulted in blockade of pulsatile LH secretion (Roseweir and Millar,2009). Furthermore, many authors demonstrated that kisspeptin level like steroid hormones fluctuates during the menstrual cycle, whereas, kisspeptin decline in the follicular phase in comparison with the preovulatory phase that suggesting kisspeptin stimulate LH secretion and enhance ovulation (Latif and Rafique 2015, Zhai *et al*., 2017).

On the other hand, kisspeptin levels are low during the early to mid-follicular phase and did not had a significant LH response to kisspeptin when follicles are underdeveloped, however, kisspeptin levels sharply increases in preovulatory women and had a significant high LH response to kisspeptin, thereby, kisspeptin levels during the periovulatory period are high and may have a potential role in predicting development of the dominant ovarian follicles (Jayasena *et al*., 2011; Zhai *et al*.,2017).

Furthermore, administration of exogenous kisspeptin increased the secretion of gonadotropin in healthy women, whereas, the inhibition kisspeptin secretion due to estrogen receptor binding with kisspeptin neurons in the arcuate nucleus and this inhibition subsequently leading to GnRH secretion decrease (Smith *et al.*, 2006; Dhillo *et al.*, 2007).

Moreover, Uenoyama and his colleagues 2021 showed that estrogen receptors α (Erα) suppresses GnRH/LH secretion that represents a negative feedback and mediates the release of preovulatory GnRH/LH surges that represents a positive feedback via its action on kisspeptin neurons in the arcuate nucleus ARC and in anteroventral periventricular nucleus AVPV in rodents , recepictively , ultimately , positive feedback initiates the LH surge associated with ovulation, in human these two types feedback exert there affects via the infundibular (arcuate) nucleus in kisspeptin neurons (Rometo *et al*., 2007 ;Hrabovszky *et al*., 2019).

Furthermore, Mittelmam-smith and his colleagues 2017 showed that progesterone also involved in the positive feedback and LH surge, kisspeptin neurons express progesterone receptors (Gal *et al*., 2016).

Moreover, Trevisan and his colleagues, 2018, suggested a new role for kisspeptin dosage as a potential predictor of follicles development and ovulation, Goto and his colleagues 2014 showed that administration of a kisspeptin antagonist resulted in shrinking of ovarian follicles and delayed ovulation, in addition, Zahi and his colleagues, 2017, mentioned that the ovulation probability be increased in association with high levels of kisspeptin (the 11th day) and LH (the 14th day) of the menstrual cycle.

Additionally, Chan and his colleagues 2012 detected that the response to kisspeptin (as measured by kisspeptin-induced GnRH-induced LH pulse) was largest in preovulatory women, intermediate in luteal-phase women and smallest in follicular-phase women, moreover, Dhillo and his colleagues 2007 observed the less LH responses in the follicular and luteal phases after exogenous administration of kisspeptin compared with preovulatory women, furthermore, Latif and Rafique 2015 also observed a significant increase in serum kisspeptin levels from the preovulatory to the luteal phase .

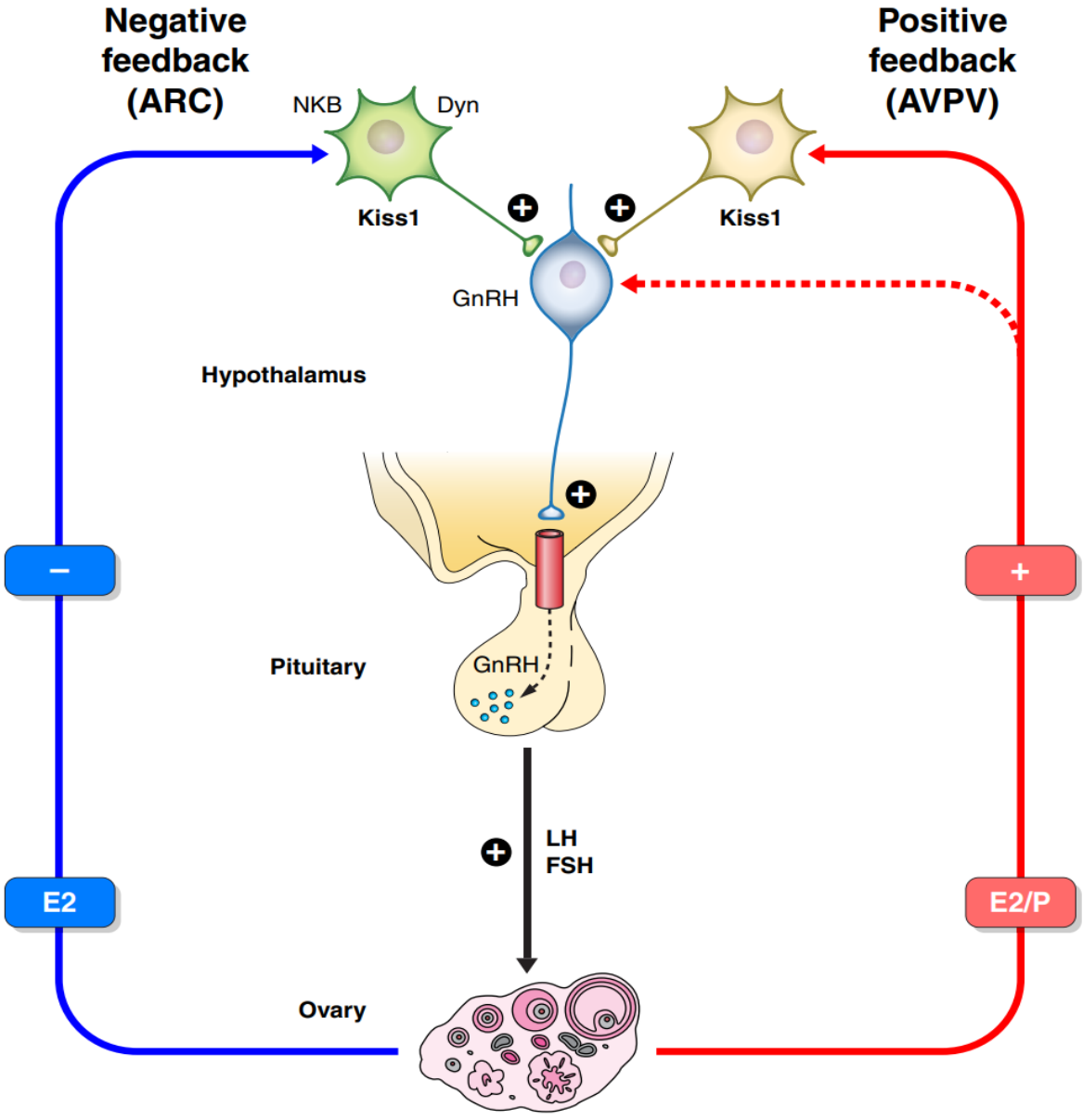


Figure 2.1: Differential regulations and actions of ARC and AVPV Kiss1 neurons in the control of GnRH in Rodent (Pinilla *et al*., 2012).

Moreover, Jamil and his colleagues 2017 thought that kisspeptin primarily functions in the hypothalamus, but also interacts between the signaling pathways of the central and peripheral reproductive systems , whereas ,many studies mentioned that kisspeptin expression in the uterus and endometrium (which regulated coordinately by the cyclical ovarian hormones, estrogen and progesterone) is absent during the proliferative phase (follicular phase) and early secretory phase (luteal phases) and becomes abundant during the late luteal phase (Baba *et al*.,2015; Hu *et al*.,2018).

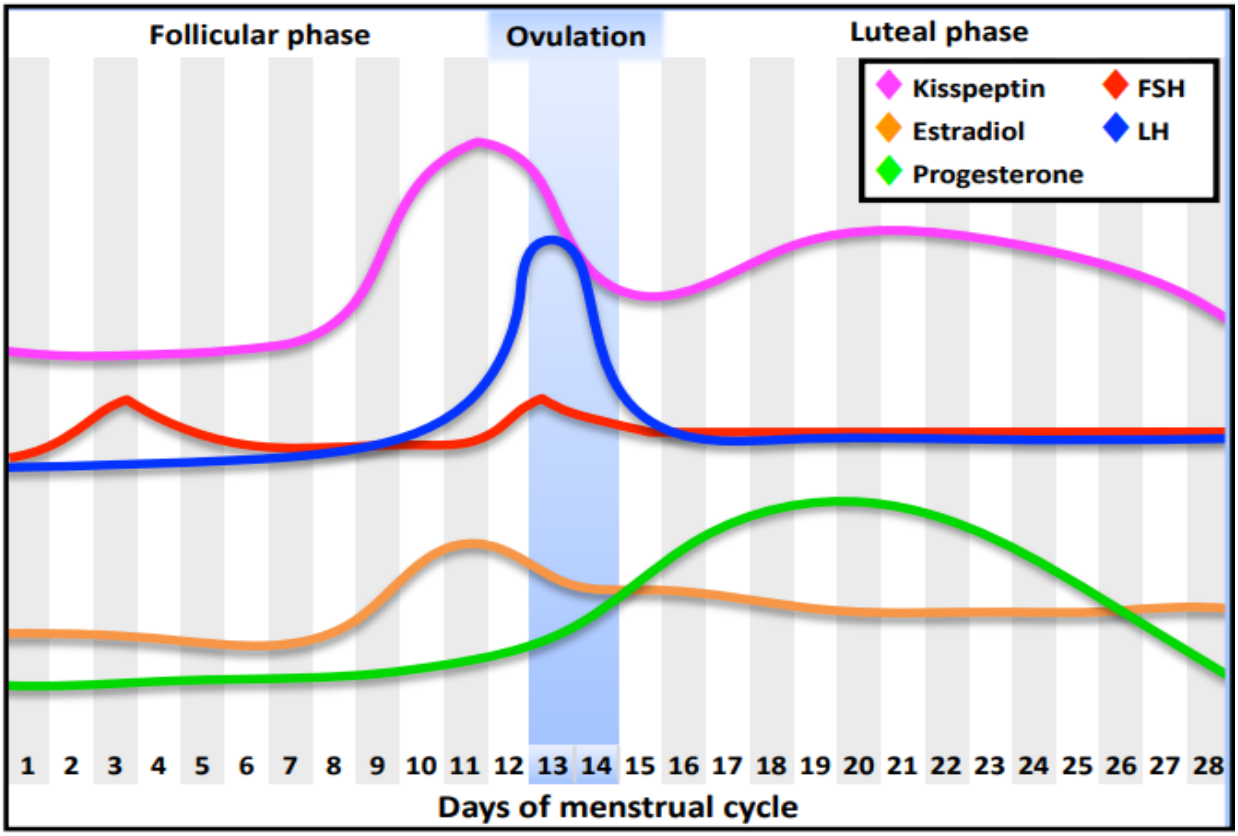


Figure 2.2: Kisspeptin levels and other reproductive hormones during menstrual cycle (Trevisan *et al*., 2018).

2.3 The menstrual Cycle and Hormonal Fluctuations

The normal ovulatory menstrual cycle consists of the follicular (proliferative) phase, ovulatory and the luteal (secretory) phase, estrogen is the primary hormone of the follicular phase, while progesterone is the primary hormone of the luteal phase (Itriyeva, 2022), during the menstrual cycle, hormones will fluctuate along with the structural changes of the ovary and uterus (Critchley *et al*., 2020).

The follicular phase begins with menstruation, whereas , estradiol levels are low and FSH levels are slightly elevated , the FSH increases which released by the anterior pituitary gland and stimulates follicular growth and estradiol production , the concentrations of FSH reach the maximum levels on the day when the dominant follicle is defined, which followed by a slow decrease during the follicular phase, reaching a nadir and then a peak just before ovulation , the estradiol along with inhibin produced by the follicles exert negative feedback on the FSH, estradiol continues to increase due to the growth of the dominant follicle until ovulation is occur (Patricio and Sergio, 2019).

In contrast, LH is low in the early follicular phase and begins to increase in the middle follicular phase due to the positive feedback by the high levels of estrogen. LH continues to increase at the peak point to complete the ovulation prosses (Itriyeva, 2022). Moreover, Duffy and his colleagues 2019 showed that the midcycle surge of LH sets in motion interconnected networks of signaling cascades to bring about rupture of the follicle and release of the oocyte during ovulation.

Patricio and Sergio,2019 found that greater amounts of estrogen secrete with the follicle matures, whereas, estrogen will increase until ovulation, then decrease slightly as inhibin increases after ovulation due to increased corpus luteum activity, while, progesterone levels remain low during the follicular phase until ovulation is reached, once ovulation has occurred, progesterone concentration will begin to increase during the luteal phase in a linear fashion.

Furthermore, progesterone exerts a negative effect on the hypothalamus, preventing the secretion of GnRH, thereby blocking the secretion of FSH and LH from the anterior pituitary gland, accompanying with the inhibin effects, as progesterone and inhibin levels increase during the luteal phase, a decrease in plasma concentrations of GnRH, FSH and LH occur (Critchley *et al*., 2020), in addition,

progesterone will stimulate endometrial growth and the lining of the uterus will eventually shed, or implantation will occur if sperm is present (Marieb and Hoehn, 2016).

Moreover, many studies pointed that once the corpus luteum degeneration, plasma concentrations of progesterone will decrease, therefore GnRH and gonadotropin released allowing for a new set of follicles to mature and new menstrual cycle to beginning (Marieb and Hoehn, 2016; Patricio and Sergio, 2019).

In addition , Phillipps and his colleagues 2020 showed that prolactin secretion influenced by increases in estradiol levels throughout the menstrual cycle , whereas , estradiol has a direct stimulatory effect on prolactin gene expression in lactotrophs and also acts in the hypothalamus to regulate tuberoinfundibular dopamine TIDA neuron , prolactin secretion remains at low basal levels during most of the menstrual cycle, but during the late follicular phase and preovulatory stage a rise in prolactin has been observed , moreover , Tanner and his colleagues 2011 stated that the prolactin levels were higher in ovulatory phase in comparison with the other two phases of the cycle and be higher also in luteal phase in comparison with the follicular phase, these rises in prolactin levels may be due to the impacts of estrogen secretion .

2.4 Oxidative stress and Anti-oxidants During Folliculogenesis and Ovulatory Prosses

In a healthy body, reactive oxygen species (ROS) and antioxidants remain balanced, when the balance is broken toward an overabundance of ROS, oxidative stress (OS) is appearing (Wang *et al*.,2022). In fact, ROS are usually considered harmful agents because their effects modify the structure and functioning of biomolecules but some ROS are also useful signaling agents (Freitas *et al*.,2017),

whereas, OS and ROS contribute to normal cellular homeostasis and ROS playing direct and indirect roles in a wide range of physiological processes (Hardy *et al*.,2021).The reproductive system is an example of the expansion of ROS physiological and regulatory roles to folliculogenesis, oocyte maturation, luteal regression and fertilization processes (Agarwal *et al*.,2012), therefore, homeostatic levels of ROS are necessary for normal oocyte maturation, fertilization and embryo development (Hardy *et al*., 2021). Moreover, Behrman and his colleagues 2001 showed that follicular ROS promote apoptosis.

Furthermore, Agarwal and his colleagues 2012 showed that a dominant oocyte that comes by different growing and developing steps during every month that targeted by an elevated ROS and inhibited by antioxidants. In contrast, the progression of meiosis II is promoted by antioxidants (Behrman *et al*.,2001), demonstrating that there is a complex relationship between ROS and antioxidants in the ovary, the increase of steroid production in the growing follicle causes an increase in cytochrome P (P450), resulting in ROS formation, whereas, ROS that produced by the pre-ovulatory follicle are considered as important inducers for ovulation (Ruder *et al*.,2009).

Moreover, Karowicz-Bilinska and his colleagues 2008 found that the H_2O_2 levels be higher during the final days of follicular phase in comparison with the luteal phase.

Furthermore, Ciani and his colleagues 2015 pointed that ROSs are generated along with ovulation that be considered as an inflammation state , thereby, ROS might be involved in the signaling cascade leading to ovulation , in addition , H_2O_2 mimicked the effect of LH in follicle rupture , via a multiple signaling pathways, including protein kinase A (PKA), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), tyrosine kinase– mediated pathways and their respective downstream and p38MAPK (Duffy *et al.*,2019).

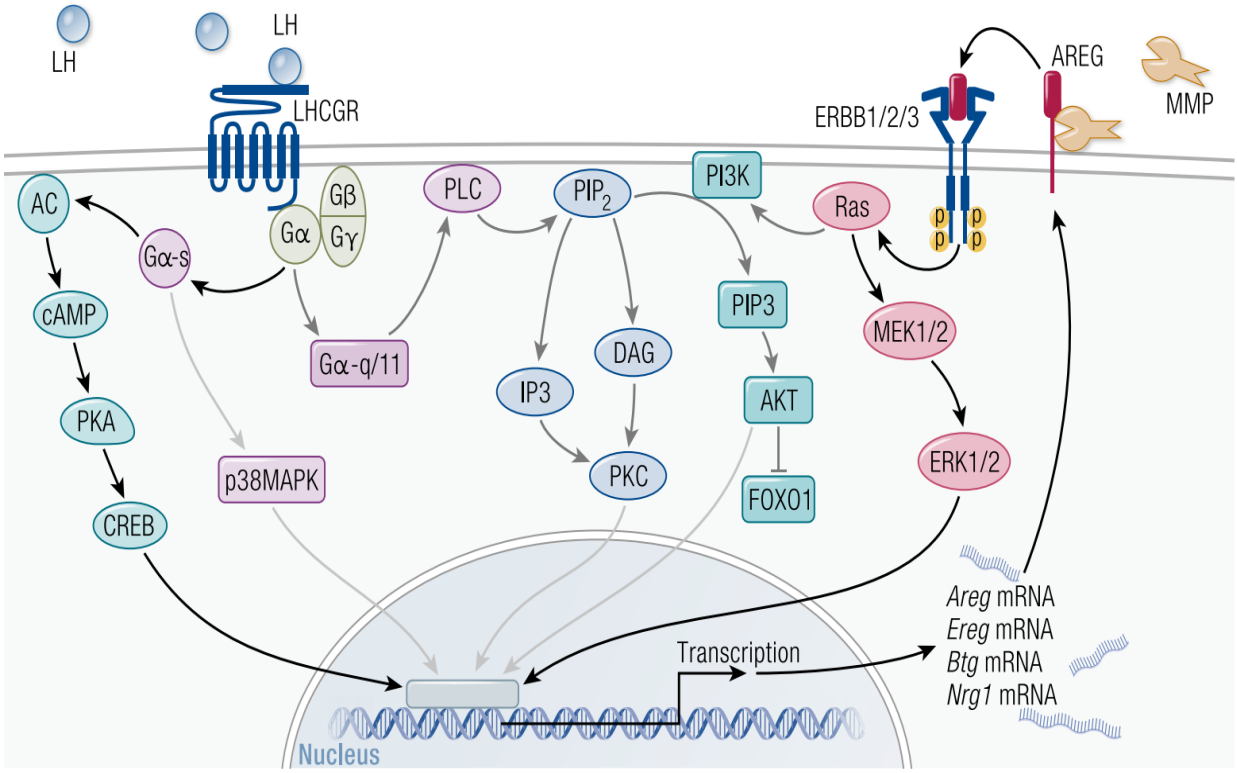


Figure 2.4: Signaling pathways activated by the LH surge in ovulatory follicles. LH activates multiple signaling pathways (Duffy *et al*.,2019).

Furthermore, an overabundance of post-LH surge inflammatory precursors generates ROS, moreover, depletion of these precursors impairs ovulation (Shkolnik *et al*.,2011).

It's worth noting that the accumulation of ROS and MDA (the product of lipid peroxidation) alleviating by kisspeptin overexpression and that enhancing the levels of the antioxidants GSH, SOD and CAT in cells, indicating that kisspeptin protects ovarian granulosa cells from oxidative stress (Sun *et al*.,2023).

On the other hand, wang and his colleagues 2017 mentioned that ROS might be induced a pathological conditions when it elevated chronically in women. Moreover, Goud and his colleagues 2008 showed that ROS such as superoxide (O_2),

hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) are molecules that are highly disruptive to cellular function, therefore, high levels of ROS production contribute significantly several diseases including those that may compromise reproduction and fertility.

On the other hand, antioxidants are indicated as factors that can maintain the balance between ROS production and clearance, a disturbance in this balance can induce pathological consequences in oocyte maturation, ovulation and fertilization (Wang *et al.*,2017) , whereas , this well-balanced redox system maintains homeostatic levels of ROS and an appropriate environment for folliculogenesis and oocyte maturation (Gupta *et al*.,2011;Ambekar *et al*.,2013; Freitas *et al*.,2017), therefore , oocytes are bath in the follicular fluid rich with the antioxidants, such as , vitamin E (α-tocopherol) , β-carotene , GSH , redox-controlling enzymes GPx , CAT and glutathione reductase (GRx) (Ambekar *et al*.,2013; Freitas *et al*.,2017).

Additionally, Ighodaro and Akinloye, 2018 showed that there are a collection of antioxidants that act to suppress or prevent the formation of ROS in cells, the key enzymes are top on this list SOD and CAT, whereas, CAT activity augmented during follicle growth and there was a lack of ROS levels in follicular fluid during follicle development, thus ensure oocyte protection from oxidative stress (Wang *et al*.,2017; Akhigbe *et al*.,2022). In addition, the increases in CAT level during follicular phase refer to their roles in follicular selection, folliculogenesis and prevention of apoptosis, whereas, CAT acts as a protective factor to neutralize H_2O_2 to maintain ROS balance and prevention of apoptosis (Paine *et al*.,2013; Wang *et al*.,2017).

Moreover, LCAT plays an important role in reverse cholesterol transport and follicular synthesis of estrogen, high LCAT activity was positively associated with antioxidant accumulation and lower LCAT activity was associated with their consumption, antioxidants are accumulated in the mature follicles to protect LCAT

from oxidative damage and promote steroidogenesis (Cruz *et al*.,2014), in addition, LCAT have a positive relationship with the fertilization rate, catalyzes lipid transporting and decreases oxidative stress that is require for the maintenance and repair of oocyte (Wu *et al*., 2016).

Furthermore, LCAT directly or indirectly interferes with several other physiological processes, including HDL antioxidant function by hydrolyzing oxidized phospholipids in oxidized LDL, adrenal steroidogenesis, insulin sensitivity, protection against obesity and platelet functions (Kunnen and Van Eck 2012).

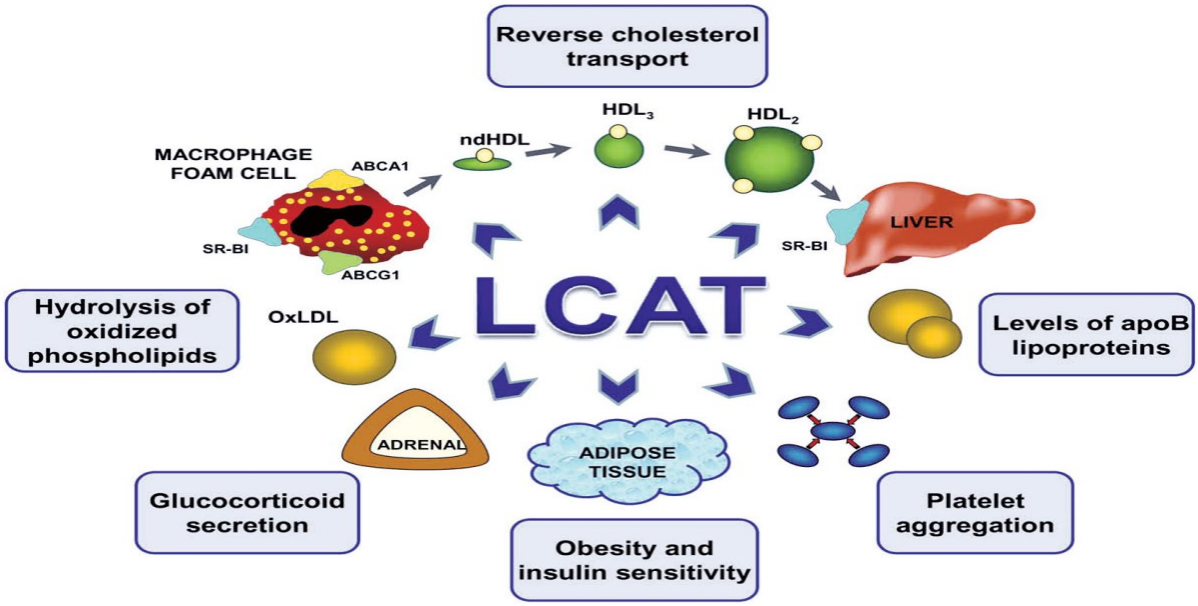


Figure 2.5: LCAT functions (Kunnen and Van Eck 2012).

2.5 Inflammation Related with Aspects of Ovulatory Process

Inflammation is the first line of defense of the organism but it is also required in many reproductive processes such as ovulation, corpus luteum development, luteolysis, uterine clearance after fertilization and postpartum, however, if excessive or persistent, inflammation can switch from a positive mechanism to a deleterious process, impairing oocyte quality and embryo development, moreover, since the female genital tract is physiologically exposed to a range of tissue injuries (such as

ovulation) and intrauterine bacterial challenges (after calving, at insemination /mating through sperm), this proves inflammation belongs to the physiology of reproduction events (Chastant and Saint-Dizier ,2019).

Whereas, inflammation is essential for successful reproduction since inflammatory (or inflammatory-like) processes that implicated in every step of cycle (ovulation, corpus luteum development, luteolysis), early pregnancy (maternal recognition of pregnancy) and later in expulsion of fetal membranes and postpartum uterine involution (Chastant and Saint-Dizier ,2019). Furthermore, ovulation is similar to an inflammatory response, the midcycle surge of LH triggers complex networks of communication cascades that result in the rupture of the follicle and release of the oocyte, the first responders to the LH surge are granulosa and theca cells which produce steroids, prostaglandins, chemokines and cytokines, which are mediators of inflammatory processes (Duffy *et al*., 2019).

On the other hand, both of formation and luteolysis of corpus luteum are inflammatory-like processes, luteolysis is considered as an acute phenomenon due to the short delay between prostaglandin F2α (PGF2α) secretion and the intra luteal immune reaction (Shirasuna *et al*., 2012). Moreover, Neuvians and his colleagues 2004; Shirasuna and his colleagues 2012 showed that the leukocytes, especially eosinophils, macrophages and T lymphocytes are recruited into the corpus luteum and the expression of pro-inflammatory cytokines is increased and made responsible for apoptosis of luteal cells, whereas, corpus luteum regresses primarily through the loss of cells by apoptosis, apoptotic luteal cells are phagocytosed by macrophages, furthermore, the large number of immune cells observed within the corpus luteum are considered essential for a rapid demise of the corpus luteum tissue.

In addition, Piccinni and his colleagues 2021 detected different cytokines, chemokines and growth factors ….i.e…interleukins , interferon-γ IFN-γ , tumor necrosis factor-α TNFα , granulocyte colony-stimulating factor G-CSF ….etc in the follicular fluids of physiological natural cycles.

Moreover , G-CSF is a multifunctional cytokine best known for stimulating the production and differentiation of neutrophils during hematopoiesis and promoting their activation with key roles in immunity and inflammatory responses (Bendall and Bradstock,2014) , recently , Noel and his colleagues 2020 showed that G-CSF is a new biomarker of oocyte quality and embryo implantation in vitro fertilization (IVF) cycles, moreover, high follicular G-CSF concentrations are associated with an increased probability of successful ovulation and implantation rate, whereas , the notable rise in G-CSF at the time of ovulation coincides with the accumulation of follicular granulocytes, most likely neutrophils, which stimulate G-CSF production by granulosa cells via paracrine interactions.

On the other hand, inflammatory aging is a new concept refers to a chronic and low-degree proinflammatory state which occurs with increasing age, termed "inflammaging" that characterized by increases in the levels of proinflammatory cytokines and contribute to organismal aging (Huang *et al*., 2019; Saavedra *et al.*,2023), moreover, Broekmans and his colleagues 2009 mentioned that inflammaging may play a relevant role in ovarian senescence , which is combined effect of both the depletion of ovarian reserve and the reduced sensitivity of gonadotropin releasing hormone, which leads to a deregulation of estrogen signaling, since macrophages expression of estrogen receptors are influenced by estrogen signaling, it is likely that these alterations at a certain point of reproductive age contribute to alter macrophages functions in ovaries, facilitating the creation of a pro-inflammatory status (Zhang *et al*., 2020).

2.6 Lipid Profile Related with The menstrual Cycle

Numerous studies have reported many differences in lipoprotein cholesterol levels throughout the menstrual cycle. The peak levels of TC and LDL were observed during the follicular phase prior to the rise and peak of estrogen, with TC and LDL levels declining during the luteal phase, corresponding to rising and peak concentrations of estrogen and progesterone, HDL levels were highest around ovulation, corresponding to high levels of estrogen, whereas triglyceride levels varied without a consistent pattern across the cycle , interestingly, variability in lipoprotein cholesterol measurements fluctuated across the cycle (Mumford *et al*.,2010; Mumford *et al*.,2011).

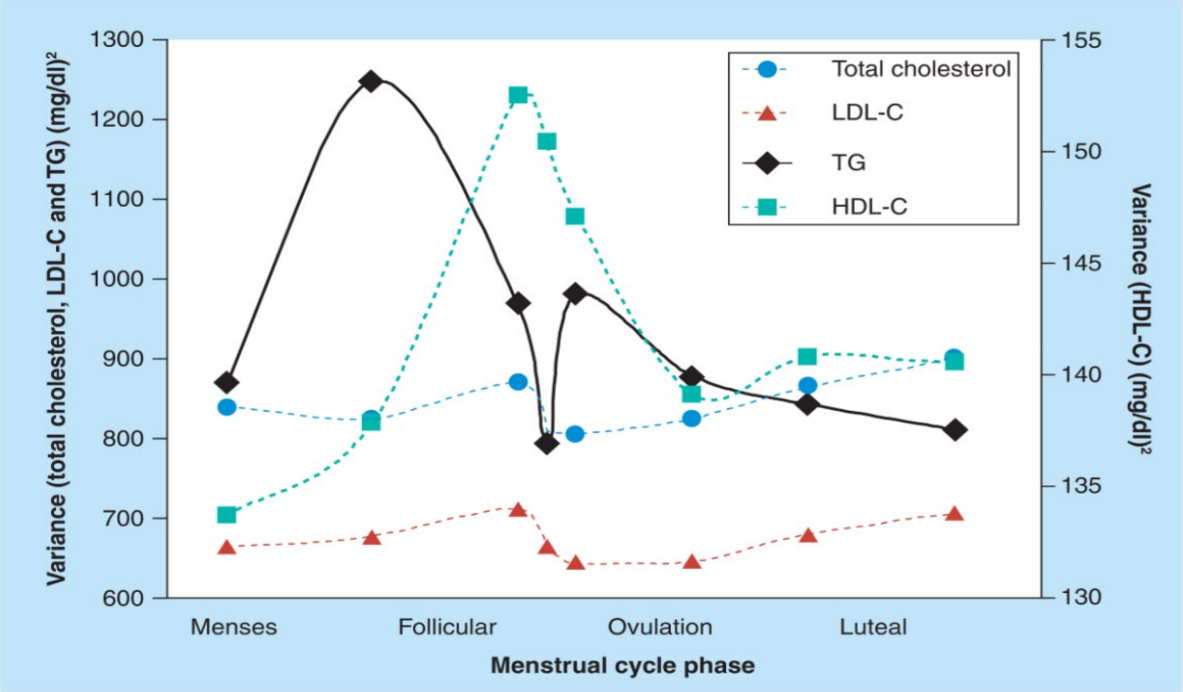


Figure 2.6: Variance of lipoprotein cholesterol measurements during different phases of the menstrual cycle (Mumford *et al*., 2011).

Furthermore, Sharma and his colleagues 2022 mentioned that TC and LDL levels increase rapidly after menses and peak during the follicular phase, followed by a decline throughout the luteal phase, the peak levels of TC and LDL during the follicular phase correspond to the rise and peak of estrogen, whereas their decline in the luteal phase corresponds to the risk and peak of progesterone , HDL levels are the highest around ovulation corresponding to the high levels of estrogen , moreover

, they recommend that clinicians take the menstrual cycle phase into account when evaluating cholesterol levels to improve the accuracy of the clinical interpretation .

Moreover , Jensen and his colleagues 2017 showed a significant increase in HDL during follicular phase and a significant decrease in TC and LDL during the luteal phase , whereas , estradiol was significantly associated with increased levels of HDL during follicular phase and ovarian effect itself (corpus luteum) was associated with significantly reduced levels of TC , HDL and LDL during the luteal phase, moreover , the corpus luteum consumes LDL to support steroidogenesis, which depletes circulating LDL during the luteal phase, regardless the independent action of estradiol and progesterone on the liver.

In addition , Panigrahi and Panda 2018 showed a significant increase in TC and LDL during the follicular phase with comparison to post ovulatory phase , while , TG decreased significantly and HDL increased significantly during the ovulatory phase in comparison with the follicular phase , whereas ,VLDL levels showed an increasing trend from follicular to post ovulatory phase , thereby , these changes might be attributed to the increase of estrogen and progesterone levels during post ovulatory phase and the effects of estrogen on hepatic lipase enzyme (HL) activity and increase in hepatic LDL receptor consequently on hepatic degradation of LDL levels .

Furthermore, Fouad Kadhuim 2020 concluded that permanence of estrogen effect is the main cause to ameliorated the lipid profile changes during different phases of the cycle in women aged (25-45 years).

In addition, lipoprotein metabolism has been affected by some estrogenic mechanisms such as the increasing of VLDL synthesis leading to subsequent decrease in LDL and increase in HDL , inhibiting hepatic lipase and lipoprotein lipase activity, up-regulating the LDL receptors (Knopp *et al*., 2005), upregulate ATP binding cassette transporter-A1 (ABCA1) that exports HDL generated from excess cellular cholesterol and Apolipoprotein-A1 (APOA1, a most important HDL protein, which enhance HDL production) (Panigrahi and Panda 2018) and suppress hepatic scavenger receptor class B Type 1 (SR-BI) expression leading to decreased hepatic cholesterol uptake from HDL (Ren *et al*.,2018), whereas, LDL and HDL play important roles in ovarian cholesterol transport, in addition, cholesterol is an important substrate for the synthesis of ovarian sex hormones and follicular development in follicular phase (Huang *et al.*,2019).

It seemed that estradiol exerts a regulatory control on the lipid metabolism, whereas, estradiol mediates the packaged of circulating fatty acid into TG rich VLDL particles by the liver, in addition, some of estradiol's protective effects in the liver are likely indirectly due to estrogen signaling adipose tissue to limit the release of serum fatty acid that is made into TG is matched with increased VLDL-TG secretion. Moreover, estradiol also increased VLDL levels by regulated the HL, which is responsible for hydrolyzing TG to chylomicrons and VLDL (Palmisano *et al.*,2017; Berad, 2019).

It's worth noting that the genes expression involved in mammal's lipid metabolism, regulates by kisspeptin via its classical pathway, whereas, kisspeptin binding with GPR54, the phospholipase C can be activated to produce phosphatidylinositol 4,5-bisphosphate (PIP2), inositol triphosphate (IP3) and diacylglycerol (DAG) and to transmit the signal in hepatocytes (Kirby *et al*., 2010). Moreover, an array of genes involved in lipid biosynthesis that activated via the effects of FSH are mediated by FSH receptors coupled to the Gai protein, as a result,

Ca^{2+} influx is stimulated, cAMP-response-element-binding protein is phosphorylated response-element-binding protein (CREB) a transcription factor known to elicit pleiotropic effects on lipid biosynthesis (Liu *et al*.,2015).

In addition, cholesterogenesis regulated by FSH that binding with hepatic FSHRs, activates the Gi2α/β-arrestin-2/Akt pathway and subsequently inhibits the binding of FoxO1 with the SREBP-2 promoter, thus preventing FoxO1 from repressing SREBP-2 gene transcription, this effect, in turn, results in the upregulation of SREBP-2, which drives HMGCR nascent transcription and de novo cholesterol biosynthesis, leading to the increase of cholesterol levels (Guo *et al*.,2019).

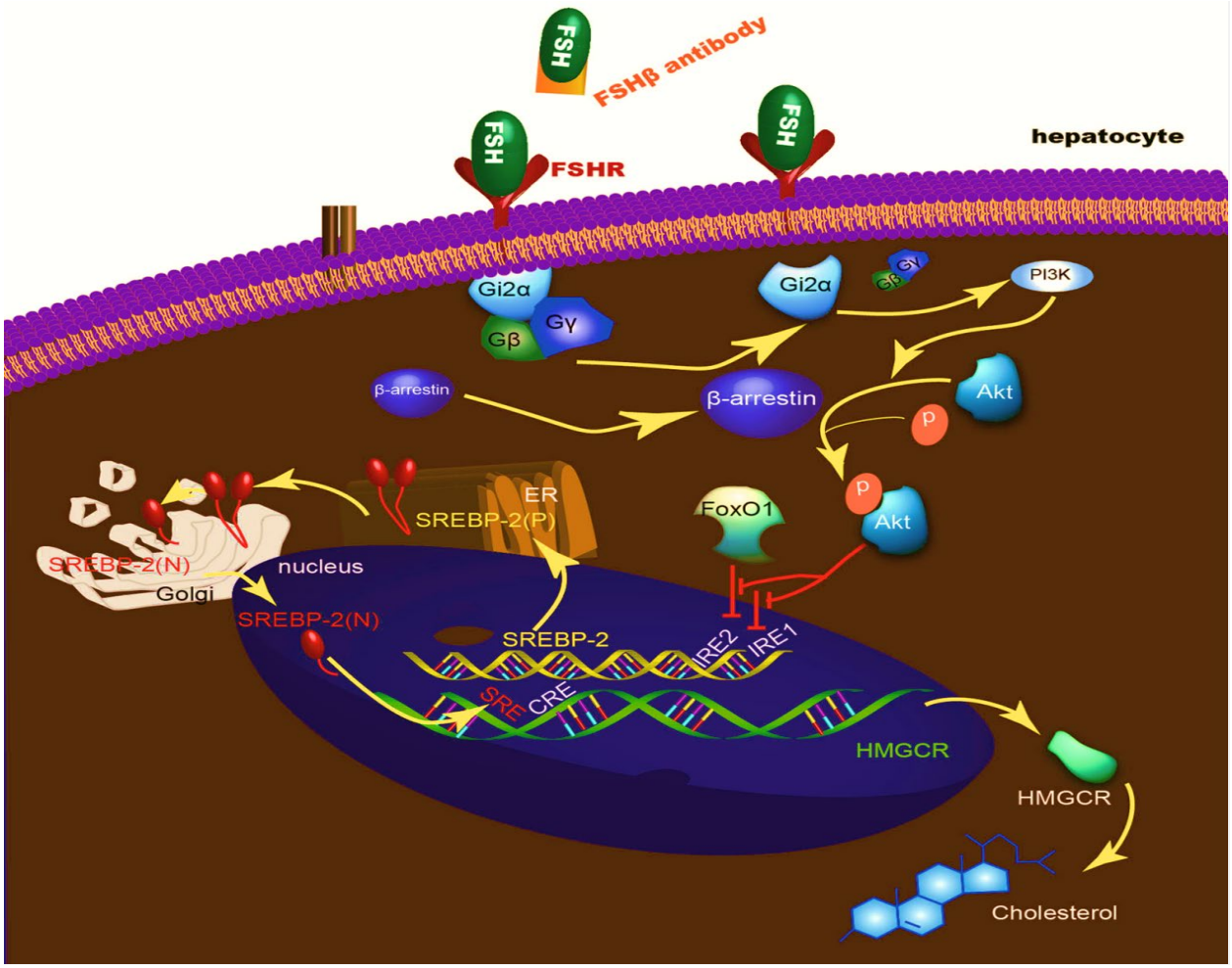


Figure 2.7: The regulation process of cholesterol biosynthesis by FSH in the liver (Guo *et al*.,2019).

Furthermore , estradiol and FSH contribute the regulating LDL levels ,whereas , estradiol controlling LDL levels by effected on the plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) that binds and controls to LDL receptors by a mechanism that involves activation of the G protein-coupled estrogen receptor (GPER) which prevents PCSK9-dependent LDLR degradation (Fu *et al*.,2020) , while , FSH involved in hepatic LDL metabolism via attenuated degradation of LDL by inhibited LDLR expression in liver , which in turn could elevate the level of serum LDL (Song *et al.*,2016).

Additionally, prolactin also participates in the regulation of hepatic lipid metabolism and exerts its biological function via cellular kinases via PI3K/AKT/STAT5 pathway (Titchenell *et al*.,2017).

Chapter Three

Materials and Methods

3.1. Materials

3.1.1. Subjects of the study:

The present study was carried out, in child and birth governmental hospital and some clinical centers in Maysan province, during October 2022 till January 2023. The sample including thirty healthy women (aged 25-45), divided (according to their ages) into two groups (15 woman / group) as the following:

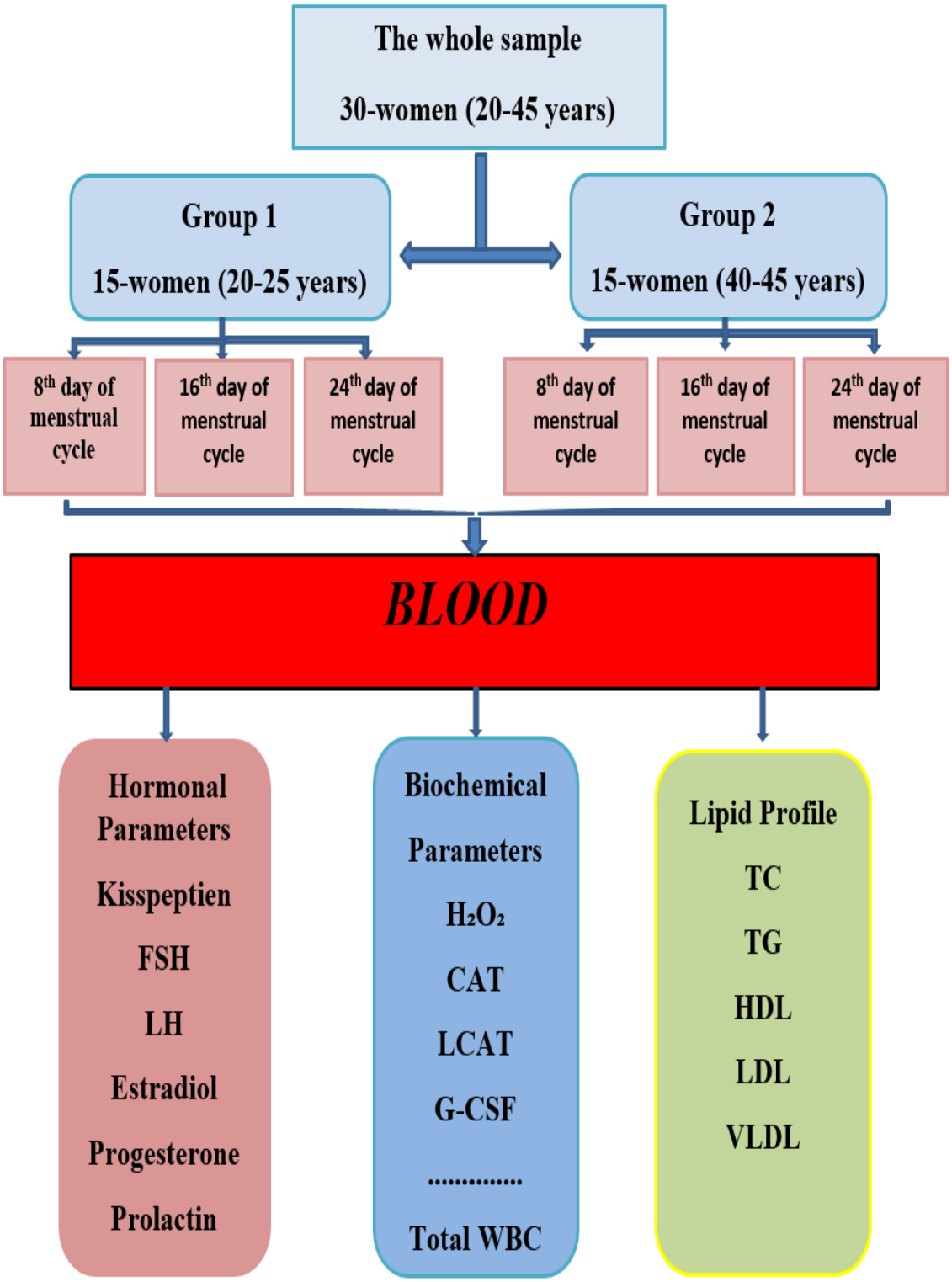
- First group: 20-25 years.
- Second group: 40-45 years.

Sample 's individuals have been diagnostic healthy women who have a regular menstrual cycle and checked medically by specialist's physician, some of these women were excluded due to their attack with diabetes, hypertension, thyroid disease, polycystic ovaries, hormonal problems, infertility and smoking, in addition to those whom taking oral contraception or any other treatment. Blood samples were taken from each woman for three days (8^{th}, 16^{th} and 24^{th}) of the menstrual cycle, to compare the studied parameters between these days for each group and also to compare these parameters between similar days for the first and second groups.

A questionnaire has been designed to obtain the actual information about the sample.

3.1.2. Experimental Design

Figure 3.1



3.1.3. Instruments and Equipments

The instruments and equipments used in this study and their origin are explained in table 3.1.

Table (3.1): The instruments and equipments used in this study.

NO	Instruments	Origin
1	Alcohol	Iraq
2	Gel and Activator tube	Jordan
3	Cotton	Turkey
4	Enzyme -Linked Immunosorbent Assays (ELISA)	Germany
5	Eppendorf tubes (1.5 ml)	Germany
6	Frozen deep freeze	Germany
7	Gloves	China
8	Gel tubes	Jordan
9	Power Spin TM Centrifuge	Japan
10	Syringe	Jordin
11	Plain tubes	China
12	Cold box	China
13	Micro pipits	Germany
14	Staining rakes	China
15	Test tubes for dilution	China
16	Tips	China

3.1.4: Laboratory Kits

The Laboratory Kits used in this study are shown in Table (3. 2) .

Table

(3. 2): Kits and their supplies.

NO	Kits	Origin
1	Kisspeptien	USA
2	FSH	France
3	LH	France
4	Estradiol	France
5	Progesterone	France
6	Prolactin	France
7	H_2O_2	China
8	CAT	China
9	LCAT	USA
10	G-CSF	USA
12	Total cholesterol (TC)	France
13	Triglyceride (TG)	France
14	High density lipoprotein (HDL)	France
15	Low density lipoprotein (LDL)	France
16	Very low-density lipoprotein (VLDL)	France

3.1.5: Diagnostic Kits

3.1.5.1 ELISA and Vidas Automated Kits:

The contents of ELISA and Vidas Kits are shown in the following:

Table (3.3): Kisspeptin ELISA Kits components.

Components	Specifications
Standard Solution (1600 ng /L)	0.5 ml x1
Pre-coated ELISA Plate	12*8 well strips x1
Standard Diluent	3 ml x1
Streptavidin-HRP	6 ml x1
Stop Solution	6 ml x1
Substrate Solution A	6 ml x1
Substrate Solution B	6 ml x1
Wash Buffer Concentrate (25x)	20 ml x1
Biotinylated Human KISS1 Antibody	1 ml x1
User Instruction	1
Plate Sealer	2 pics

Table (3.4): FSH Vidas Kits components.

Components	Specifications
FSH Strips	60
FSH SPRs	2 x 30
FSH Control	1 x 3 ml (lyophilized)
FSH Calibrator	3 x 2 ml (lyophilized)
FSH Diluent	1 x 3 ml (liquid)

Table (3.5): LH Vidas Kits components.

Components	Specifications
LH Strips	60
LH SPRS	2 x 30
LH Control	1 x 3 ml (lyophilized)
LH Calibrator	3 x 2 ml (lyophilized)
LH Diluent	1 x 3 ml (liquid)

Table (3.6): Estradiol Vidas Kits components.

Components	Specifications
E2 II Strips	60
E2 II SPRs	2 x 30
E2 II Control	1 x 3 ml (liquid)
E2 II Calibrator	2 x 4 ml (liquid)

Table (3.7): Progesterone Vidas Kits components.

Components	Specifications
PRG Strips	60
PRG SPRs	2 x 30
PRG Control	1 x 3 ml (lyophilized)
PRG Calibrator	2 x 4 ml (lyophilized)

Table (3.8): Prolactin Vidas Kits components.

Components	Specifications
PRL Strips	60
PRL SPRs	2 x 30
PRL Control	1 x 3 ml (lyophilized)
PRL Calibrator	3 x 2 ml (lyophilized)
Diluent	1 x 3 ml (liquid)

Table (3.9): H_2O_2 ELISA Kits components.

Components	Specifications
Coated ELISA Plate	12-Well * 8 Tubes
Standard Dilution	3 ml
Chromogen Solution A	6 ml
Chromogen Solution B	6 ml
Streptavidin -HRP	6 ml
Standard Solution (80 pg/ml)	0.5 ml
Washing Concentrate (30 x)	20 ml
Instruction	1
Seal Plate membrane	2
Hermetic bag	1
Stop Solution	6 ml
Anti H_2O_2 antibodies labeled with biotin	1 ml

Table (3.10): CAT ELISA Kits components.

Components	Specifications
Coated ELISA Plate	12-Well * 8 Tubes
Standard Dilution	3 ml
Chromogen Solution A	6 ml
Chromogen Solution B	6 ml
Streptavidin -HRP	6 ml
Standard Solution (800 KU/L)	0.5 ml
Washing Concentrate (30 x)	20 ml
Instruction	1
Seal Plate membrane	2
Hermetic bag	1
Stop Solution	6 ml
Anti CAT antibodies labeled with biotin	1 ml

Table (3.11): LCAT ELISA Kits components.

Components	Specifications
Standard Solution (960U/L)	0.5 ml x 1
Pre-coated ELISA Plate	12 * 8 well strips x 1
Standard Diluent	3 ml x 1
Streptavidin -HRP	6 ml x 1
Stop Solution	6 ml x 1
Substrate Solution A	6 ml x 1
Substrate Solution B	6 ml x 1
Wash Buffer Concentrate (25 x)	20 ml x 1
Biotinylated Human LCAT Antibody	1 ml x 1
User Instruction	1
Plate Sealer	2 Pics

Table (3.12): G-CSF ELISA Kits components.

Components	Specifications
Pre-Coated Microplate Standard (lyophilized)	12 strips x 8 wells
Standard (lyophilized)	2 vials
Biotinylated Antibody (100x)	120 ul
Streptavidin-HRP (100x)	120 ul
Standard /Sample Diluent Buffer	20 ml
Biotinylated Antibody Diluent	12 ml
HRP Diluent	12 ml
Wash Buffer (25x)	20 ml
TMB Substrate Solution	9 ml
Stop Reagent	6 ml
Plate Covers	2 pieces

3.1.5.2 Bio-System Spectrophotometer Automated Kits:

The contents of Bio-Systems Kits are listed as the following:

a- Total cholesterol Bio-Systems kits components

Reagent 10x50 mL. Pipes 35 mmol/L, sodium cholate 0.5 mmol/L, phenol 28 mmol/L, cholesterol esterase > 0.1 U/m L, peroxidase > 0.8 U/m L,4aminoantipyrine 0.5 mmol/L, Ph 7.0, Biochemistry calibrator human (Biosystems cod.18044).

b-Triglycerides Bio-Systems kits components

Reagent 10x50 mL. Pipes 45 mmol/L, magnesium acetate 5 mmol/L,4chlorophenol 6 mmol/L, lipase >100 U/m L, glycerol kinase > 1.5 U/m L, glycerol-3-phosphate oxidase > 4 mmol/L, peroxidase> 0.8 U/m L,4aminoantipyrine 0.75 mmol/L, ATP 0.9 mmol/L, Ph 7.0, Biochemistry calibrator human (Biosystems cod.18044).

c-High density lipoprotein cholesterol Bio-Ststems kits components

A. Reagent. 3x 20 m L. Good's buffer, cholesterol oxidase < 1 U/m L, peroxidase < 1 U/ m L, N, N-bis(4-sulfobutyl)-m-toluidine (DSB m T) 1mmol/L.
B. Reagent. 1x20 m L. Good's buffer, cholesterol esterase < 1.5 U/ m L,4-aminoantipyrine 1 mmol/L, ascorbate oxidase< 3.0 KU/L, detergent, Biochemistry calibrator human (Biosystems cod.18044).

d-Low density lipoprotein cholesterol Bio-Ststems kits components

A. Reagent.3x20m L.MES buffer>/L, cholesterol esterase < 1.5 U/m L, cholesterol oxidase < 1.5 U/m L ,4-aminoantipyrine 0.5 mmol/L, ascorbate oxidase < 3.0 U/L, peroxidase > 1 U/m L, detergent, pH6.3.
B. Reagent. 1x20 m L. MES buffer > 30 mmol/L, N, N-bis (4sulfobutyl)-m-toluidine (DSBm T)1 mmol/L, detergent, Ph 6.3.

3.2 Methods

3.2.1 Obtaining Blood Samples:

Venous blood samples (8-10 mL) were drawn at 9 - 11 a.m., the blood samples were left for 20 minutes to clot at room temperature, to get the serum which separated by centrifugation at 3000 rpm for 15 min, to assay all the parameters for the current study, serum was transferred into labeled plain tube and stored at -20 C˚until used for evaluation of hormones and the other parameters.

3.2.2 Determination of Hormones Assays

3.2.2.1 Determination of Kisspeptin -1 Hormone

Kisspeptin is evaluate by using enzyme-linked-immunosorbent-assay (ELISA) system, with human Kisspeptin kit.

Assay Principle

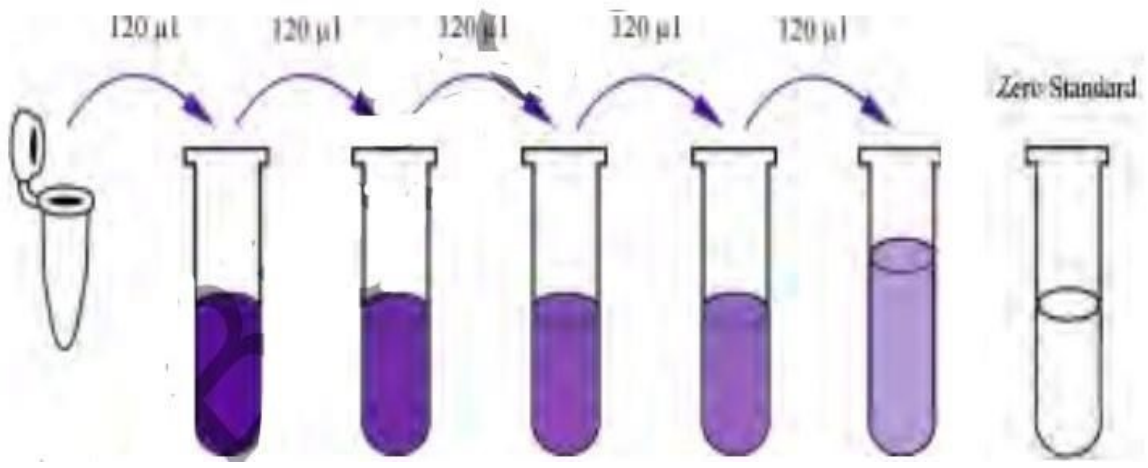
This kit is an Enzyme – Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human KISS1 antibody. KISS1 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human KISS1 Antibody is added and binds to KISS1 in the sample. Then StreptavidinHRP is added and binds to the Biotinylated KISS1 antibody. After incubation unbound Streptavidin -HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human KISS1. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

The procedure:

Reagent Preparation

1. All reagents should be brought to room temperature before use.
2. Standard Reconstitute the 120µl of the standard (1600ng/L) with 120µl of standard diluent to generate an 800ng/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (800ng/L) 1:2 with standard diluent to produce 400ng/L, 200ng/L, 100ng/L and 50ng/L solutions. Standard diluent serves as the zero standard (0 ng/L). Any remaining solution should be frozen at -20℃ and used within one month. Dilution of standard solutions suggested are as follows:

800ng/L, Standard No.5, 120µl Original Standard + 120µl Standard Diluent
400ng/L, Standard No.4, 120µl Standard No.5 + 120µl Standard Diluent
200ng/L, Standard No.3, 120µl Standard No.4 + 120µl Standard Diluent
100ng/L, Standard No.2, 120µl Standard No.3 + 120µl Standard Diluent
50ng/L, Standard No.1, 120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
1600ng/L	800ng/L	400ng/L	200ng/L	100ng/L	50ng/L

3. Wash Buffer Dilute 20ml of Wash Buffer Concentrate 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

The procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Add 50µl standard to standard well. Note: Don't add antibody to standard well because the standard solution contains biotinylated antibody.
4. Add 40µl sample to sample wells and then add 10µl anti-KISS-1 antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.

6. Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50µl Stope Solution to each well, the blue color will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

3.2.2.2 Determination of Fertility Hormones (FSH, LH, Estradiol, Progesterone and Prolactin).

Determinate by using Vidas system, with human FSH, LH, Estradiol, Progesterone and Prolactin) kits.

The procedure

1. Remove the necessary components from kit, and store the remain components at (2 – 8 °C).
2. Allow time for the components, to warm up to room temperature (approximately 30 minutes).
3. For each sample: control or calibrator, to be tested, use one " FSH, LH, Estradiol, Progesterone and Prolactin " strip, and one " FSH, LH, Estradiol, Progesterone and Prolactin " SPR. After an appropriate SPRs have been withdrawn, making sure the storage pouch, has been carefully resealed.
4. The test was identified by the (FSH, LH, Estradiol, Progesterone and (Prolactin) code, on an instrument. The calibrator must be identified by S1, and tested in duplicate, if the control tested, it should be to identified by C1.
5. Label the (FSH, LH, Estradiol, Progesterone and Prolactin) Reagent Strips with the appropriate sample ID numbers if necessary.

6. Using a vortex type mixer, combine the calibrator, control, and sample.
7. For this test; the calibrator, control and sample tests portion are (200 µl).
8. Place the (FSH, LH, Estradiol, Progesterone and Prolactin) Reagent- Strips and SPRs in an instrument's appropriate positions. Check that the color labels on the SPRs and the Reagent- Strips match assay code.
9. As recommended in the Operator's-Manual of vidas of biomatrix, begin the assay processing. The equipment performs all of the assay processes automatically.
10. After pipetting, re-close the vials, and bring them to a necessary temperature.
11. The assay will take about (40) minutes to complete. Removed the SPRs, and strips from an instrument once an assay is finished.
    (Bardin *et al*.,1981; Badonnel and Coll,1994; Darai *et al*.,1995; Sapin and Simon 2001).

Assay range (FSH)	Follicular :3.0-12.0 mlU/ml Luteal:1.5-7.0 mlU/ml. Ovulation:6.3-24.0 mlU/ml.
Assay range (LH)	Follicular: 2.0-8.0 mlU/ml. Luteal:0.2-6.5 mlU/ml. Ovulation:9.6-80.0 mlU/ml
Assay range (Estradiol)	Follicular:18-147pg/ml Luteal:43-214 pg/ml Ovulation:93-575 pg/ml
Assay range (Progesterone)	Follicular:2.0-8.0ng/ml Luteal:0.2-6.5 ng/ml Ovulation:9.6-80.0 ng/ml
Assay range (Prolactin)	5-25 ng/ml

3.2.3 Determination of Biochemical Assay

3.2.3.1 Determination of Human Hydrogen Peroxide(H_2O_2)

H_2O_2 is evaluate by using enzyme-linked-immunosorbent-assay (ELISA) system, with human H_2O_2 kit.

Assay Principle

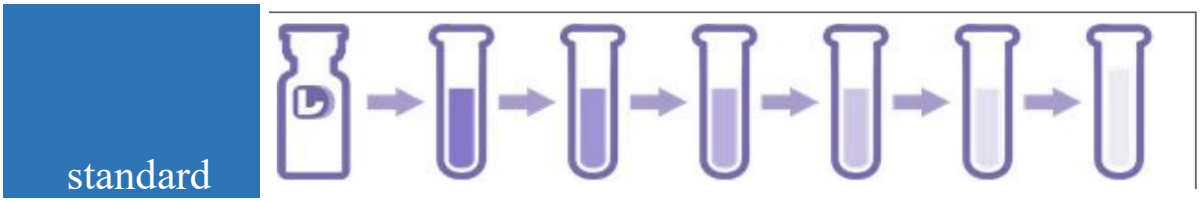
This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Hydrogen Peroxide (H_2O_2). Add Hydrogen Peroxide(H_2O_2) to the wells, which are precoated with Hydrogen Peroxide(H_2O_2) monoclonal antibody and then incubate. After that, add anti H_2O_2 antibodies labeled with biotin to unite with streptavidin HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B.

Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Hydrogen Peroxide (H_2O_2) are positively correlated.

The procedure:

1.Dilution of standard solutions: (This kit has a standard of original concentration, which could be diluted in small tubes by user independently following the instruction):

400U/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
200U/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
100U/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
50U/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
25U/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Tube	standard	S5	S4	S3	S2	S1
pg/ml	80	40	20	10	5	2.5

2.The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.

3.Sample injection: 1) Blank well: Add only Chromogen solution A and B, and stop solution. 2) Standard solution well: Add 50µl standard and streptavidin-HRP 50µl. 3) Sample well to be tested: Add 40µl sample and then 10µl H_2O_2 antibodies, 50µl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37℃.

4.Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.

5.Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.

6.Color development: Add 50µl chromogen solution A firstly to each well and then add 50µl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.

7.Stop: Add 50µl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).

8.Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

9.According to standards' concentrations and the corresponding OD values, calculate the linear regression equation of the standard curve. Then according to the OD value of samples, calculate the concentration of the corresponding sample. Special software could be employed to calculate as well.

3.2.3.2 Determination of Human Catalase (CAT)

CAT is evaluating by using enzyme-linked-immunosorbent-assay (ELISA) system, with human CAT kit.

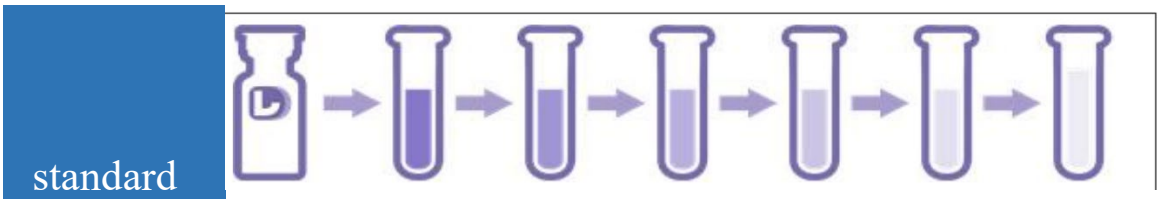
Assay Principle

This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Catalase (CAT). Add Catalase (CAT)to the wells, which are pre-coated with Catalase (CAT)monoclonal antibody and then incubate. After that, add anti CAT antibodies labeled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Catalase (CAT) are positively correlated.

The procedure:

1. Dilution of standard solutions: (This kit has a standard of original concentration, which could be diluted in small tubes by user independently following the instruction.):

400U/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
200U/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
100U/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
50U/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
25U/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Tube	standard	S5	S4	S3	S2	S1
KU/L	800	400	200	100	50	2.5

1. The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.
2. Sample injection: 1) Blank well: Add only Chromogen solution A and B, and stop solution. 2) Standard solution well: Add 50μl standard and streptavidin-HRP 50μl. 3) Sample well to be tested: Add 40μl sample and then 10μl CAT antibodies, 50μl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37℃ for 60 minutes.
3. Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.
4. Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.
5. Color development: Add 50μl chromogen solution A firstly to each well and then add 50μl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.

6. Stop: Add 50µl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).
7. Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.
8. According to standards' concentrations and the corresponding OD values, calculate the linear regression equation of the standard curve. Then according to the OD value of samples, calculate the concentration of the corresponding sample. Special software could be employed to calculate as well.

3.2.3.3 Determination of Human Lecithin Cholesterol Acyltransferase (LCAT)

LCAT is evaluate by using enzyme-linked-immunosorbent-assay (ELISA) system, with human LCAT kit.

Assay Principle

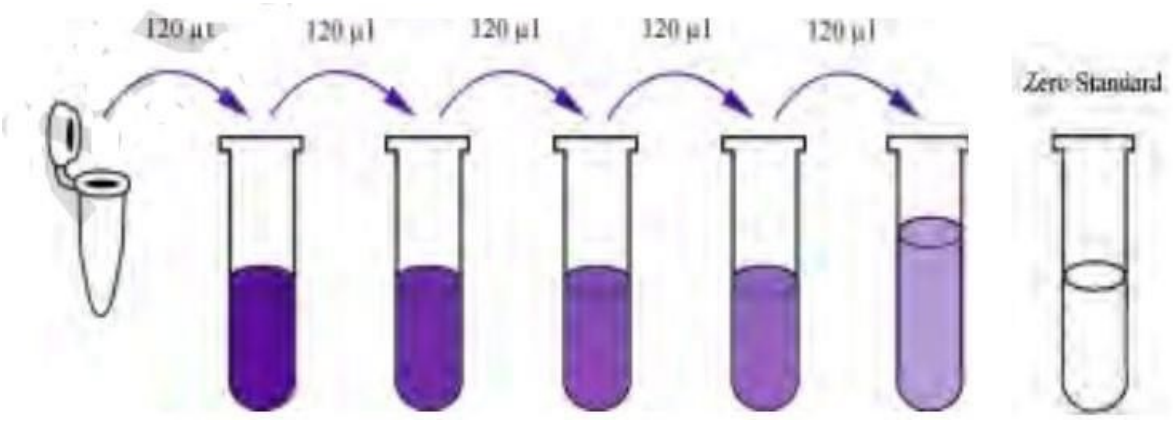
This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with human LCAT antibody. LCAT present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human LCAT Antibody is added and binds to LCAT in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated LCAT antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human LCAT. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent Preparation

1.All reagents should be brought to room temperature before use.

2.Standard Reconstitute the 120µl of the standard (960U/L) with 120µl of standard diluent to generate a 480U/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (480 U/L) should be serially diluted by 1:2 with the standard diluent to create solutions with volumes of 240 U/L, 120 U/L, 60 U/L, and 30 U/L. The zero standard (0 U/L) is the standard diluent. Any solution that is left over has to be frozen at -20°C and utilized within a month. The following are proposed dilutions for standard solutions:

480U/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
240U/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
120U/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
60U/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
30U/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
960U/L	480U/L	240U/L	120U/L	60U/L	30U/L

3.Wash Buffer Dilute 20ml of Wash Buffer Concentrate 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

The procedure:

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Add 50µl standard to standard well. Note: Don't add antibody to standard well because the standard solution contains biotinylated antibody.
4. Add 40µl sample to sample wells and then add 10µl anti-LCAT antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

3.2.3.4 Determination of Human Colony Stimulating Factor (G- CSF)

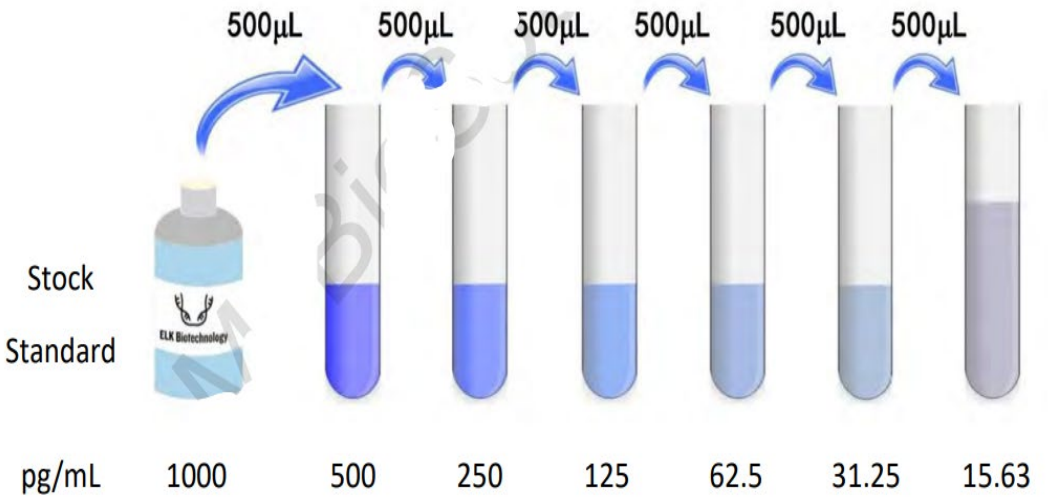
G-CSF is evaluated by using enzyme-linked-immunosorbent-assay (ELISA) system, with human G-CSF kit.

The principle

The test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Colony Stimulating Factor, Granulocyte (G-CSF). Standards or samples are added to the appropriate microtiter plate wells then with a biotin-conjugated antibody specific to Colony Stimulating. Factor, Granulocyte (G-CSF). Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain Colony Stimulating Factor,

Granulocyte (G-CSF), biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of Colony Stimulating Factor, Granulocyte (G-CSF) in the samples is then determined by comparing the OD of the samples to the standard curve.

1. Bring all kit components and samples to room temperature (18-25°C) before use.
2. If the kit will not be used up in one time, please only take out strips and reagents for present
experiment, and save the remaining strips and reagents as specified.
3. Dilute the 25x wash buffer into 1x working concentration with double steaming water.
4. Standard working solution-Reconstitute the Standard with 1.0mL of Standard Diluent, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the standard in the stock solution is 1000 pg/mL. Please prepare 7 tubes containing 0.5mL Standard Diluent and use the diluted standard to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL, 15.63 pg/mL, and the last EP tubes with Standard Diluent is the Blank as 0pg/mL. In order to guarantee the experimental results validity, please use the new standard solution for each experiment.



5. Biotinylated Antibody and Streptavidin-HRP: Briefly spin or centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to the working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6. TMB substrate - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

The procedure

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100 µL each of standard working solution (read Reagent Preparation), or 100 µL of samples into the appropriate wells. Cover with the Plate sealer. Incubate for 80 minutes at 37°C.

2. Remove the liquid of each well. Aspirate the solution and wash with 200 µL of 1× Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

3. Add 100 µL of Biotinylated Antibody working solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.
4. Repeat the aspiration, wash process for total 3 times as conducted in step 2.
5. Add 100 µL of Streptavidin-HRP working solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.
6. Repeat the aspiration, wash process for total 5 times as conducted in step 2.
7. Add 90 µL of TMB Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes).
Protect from light. The liquid will turn blue by the addition of TMB Substrate Solution
8. Add 50 µL of Stop reagent to each well. The liquid will turn yellow by the addition of Stop reagent. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop reagent should be the same as that of the TMB Substrate Solution.
9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

3.2.3.4 Total WBC Count

The number of WBC counted by automated cell counters, by ABX Micros ES 60, HORIBA Medical.

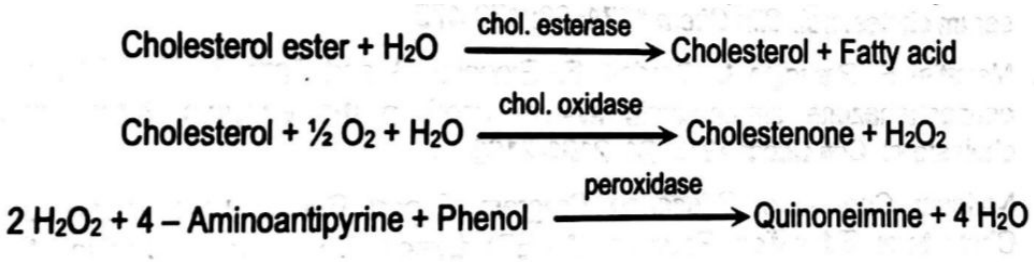
3.2.3.6 Determination of Lipid Profile (TC, TG, HDL, LDL, VLDL)

3.2.3.6.1 Determination of Total Cholesterol (TC)

TC is evaluated by using spectrophotometrically automated, with human TC kit.

Assay Principle

Free and esterified cholesterol in the sample originates, by means of the coupled reaction described below, a colored complex that can be measured by spectrophotometry (Allain *et al*.,1974; Meiattini *et al*.,1978).



Reagent Preparation

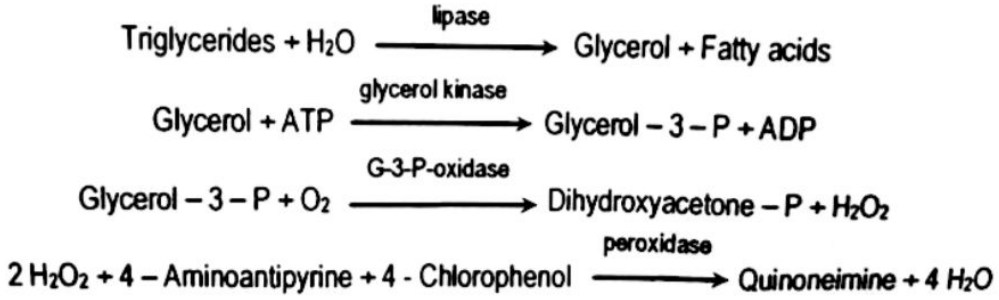
Reagent is provided ready to use.

3.2.3.6.2 Determination of Triglycerides (TG)

TG is evaluated by using spectrophotometrically automated, with human C kit.

Assay Principle

Triglycerides in the sample originates, by means of the coupled reaction described below, a colored complex that can be measured by spectrophotometry (Bucolo and David ,1973; Fossati and Prencipe ,1982).



Reagent preparation

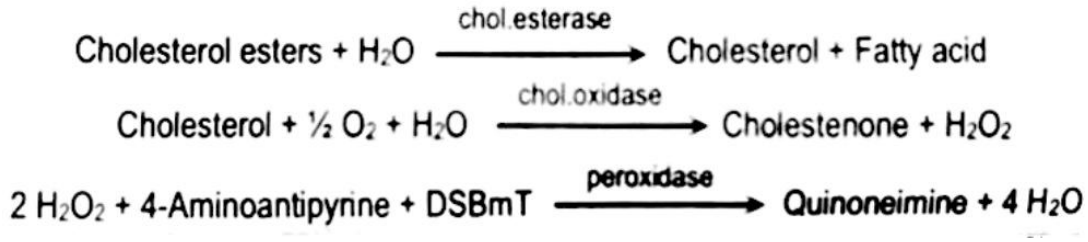
Reagent is provided ready to use.

3.2.3.6.3 Determination of High-Density Lipoprotein (HDL)

HDL is evaluated by using spectrophotometrically automated, with human HDL kit .

Assay Principle

The cholesterol from low density lipoproteins (LDL), very low-density lipoproteins (VLDL) and chylomicrons, is broken down by the cholesterol oxidase in an enzymatic accelerated non-color forming reaction. The detergent present in the reagent B, solubilizes cholesterol from high density lipoprotein (HDL) in the sample. The HDL cholesterol is then spectrophotometrically measured by means of the coupled reaction described below (Warnick *et al*.,2001)



Reagent Preparation

Reagents are provided ready to use.

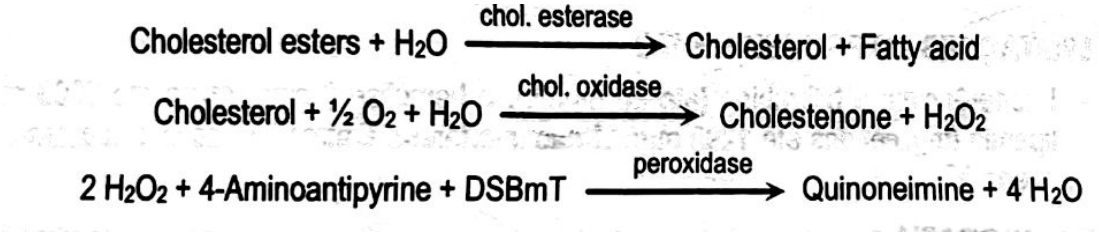
3.2.3.6.4 Determination of Low-Density Lipoprotein (LDL)

LDL is evaluated by using spectrophotometrically automated, with human LDL kit and /or by using the following equation:

LDL=TC(HDL+TG/5)

Assay Principle

A specific detergent solubilizes the cholesterol from high density lipoproteins (HDL), very low-density lipoproteins (VLDL) and chylomicrons. The cholesterol esters are broken down by cholesterol esterase and cholesterol oxidase in a non-color forming reaction. The second detergent, present in the reagent B, solubilizes cholesterol from low density lipoproteins (LDL)in the sample. The LDL cholesterol is then spectrophotometrically measured by means of the coupled reactions described below (Nauck *et al*.,2002).



Reagent Preparation

Reagents are provided ready to use.

3.2.3.6.5 Determination of Very Low-Density Lipoprotein (VLDL)

VLDL is evaluated by using spectrophotometrically automated, by using the following equation:

VLDL= TG/5

Assay Principle

Estimation of the concentration of very low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. (Friedewald *et al*.,1972). The VLDL cholesterol is then spectrophotometrically measured by means of the coupled reactions.

3.3 Statistical Analysis

The results are expressed as mean ± Standard Deviation (SD), this analysis was performed by IBM SPSS statistics, version 26 (IBM Co., Armonk, NY, USA) , by one-way Analysis of Variance (ANOVA), followed by Duncan's test and by t-test under the significant level ($p \leq 0.05$) (Steel *et al*., 1997).

Chapter Four

Results

4.1 Hormonal Parameters

4.1.1 kisspeptin

First Group:

Results revealed that kisspeptin increased non-significantly in the 16th day (274.86 ± 19.202 pg/ml) in comparison with the 8th day (229.06 ± 15.986 pg/ml) and significantly ($p \leq 0.05$) with the 24th day (222.4 ± 15.767 pg/ml). Table 4.1.1, figure 4.1.1.1.

Second Group:

Results revealed that kisspeptin increased non-significantly in the 16th day (298.80 ± 28.59 pg/ml) in comparison with the 8th day (251.66 ± 28.19 pg/ml) and significantly ($p \leq 0.05$) with the 24th day (239.13 ± 27.37pg/ml). Table 4.1.1, figure 4.1.1.1.

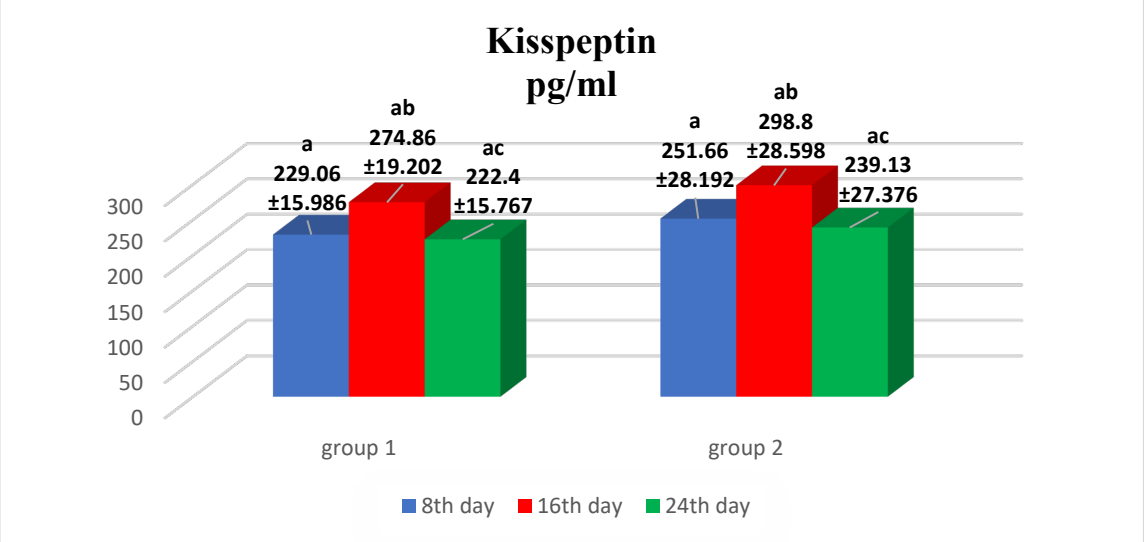


Figure (4.1.1.1): The levels of kisspeptin changes during different phases of menstrual cycle in both groups.

Between Groups:

Kisspeptin increased significantly ($p \leq 0.05$) in both 8th and 16th days in the second group in comparison with the first group. And non-significantly in the 24th day between first and sconed groups. Table 4.1.2, figure 4.1.1.2.

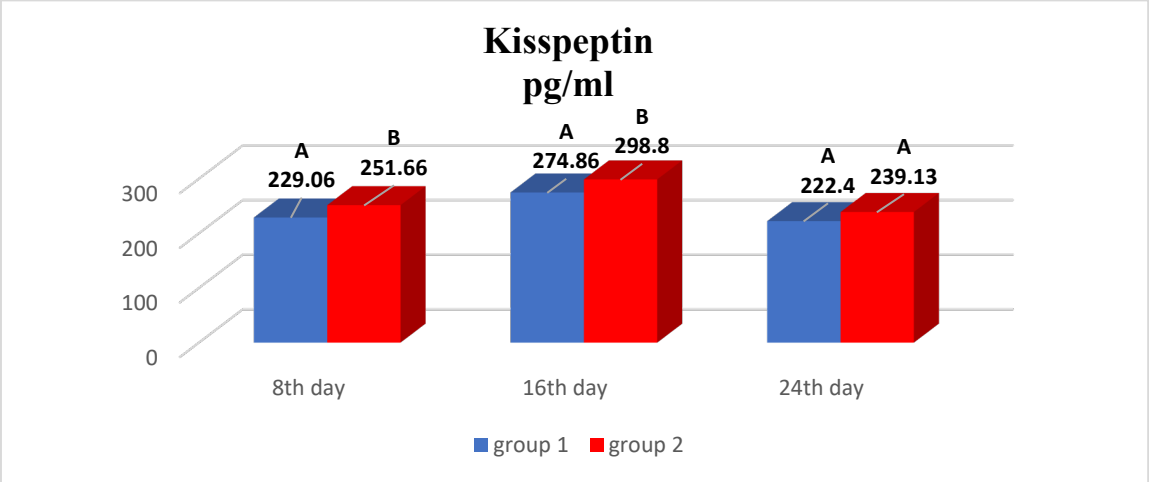


Figure (4.1.1.2): The levels of kisspeptin for similar days in different phases between both groups.

4.1.2 FSH

First Group:

Results revealed that FSH increased non-significantly in the 8th day (5.386 ± 0.883 mlu/ml) in comparison with the 16th day (4.406 ± 0.717 mlu/ml) and significantly ($p \leq 0.05$) with the 24th day (3.186 ± 0.680 mlu/ml). Table 4.1.1, figure 4.1.2.1.

Second Group:

Results revealed that FSH increased significantly ($p \leq 0.05$) in the 8th day (6.173±0.459 mlu/ml) in comparison with both the 16th day (4.973 ±0.367 mlu/ml) and the 24th day (3.740 ± 0.421 mlu/ml). Table 4.1.1, figure 4.1.2.1.

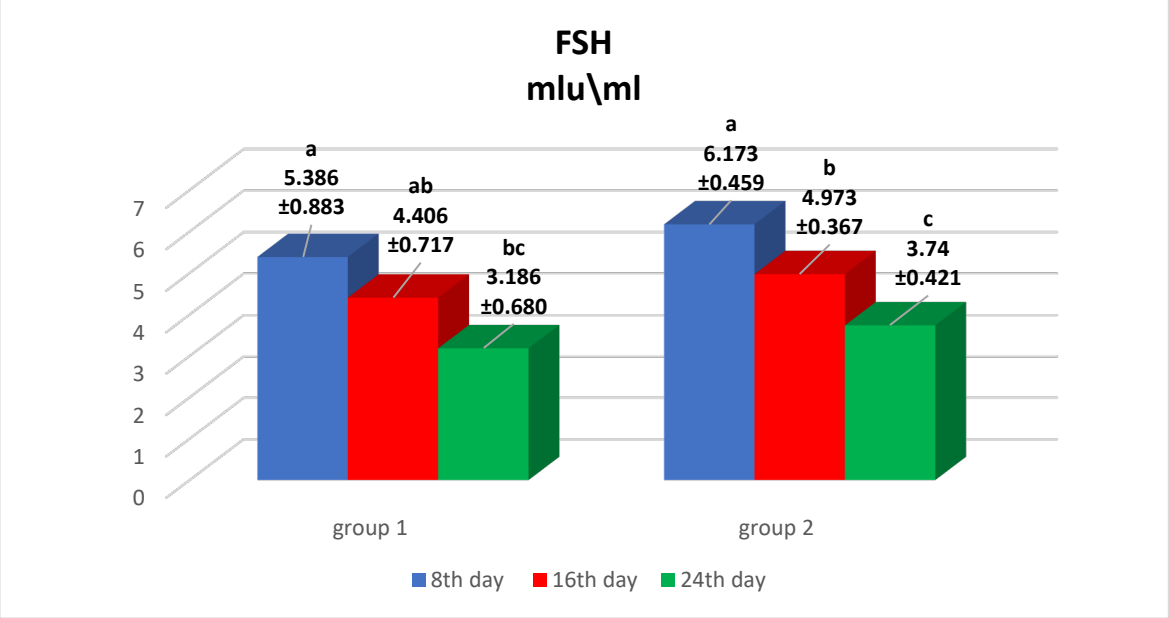


Figure (4.1.2.1): The levels of FSH changes during different phases of menstrual cycle in both groups.

Between Groups:

FSH increased significantly ($p \leq 0.05$) in the second group in comparison with the first group for all similar days. Table 4.1.2, figure 4.1.2.2.

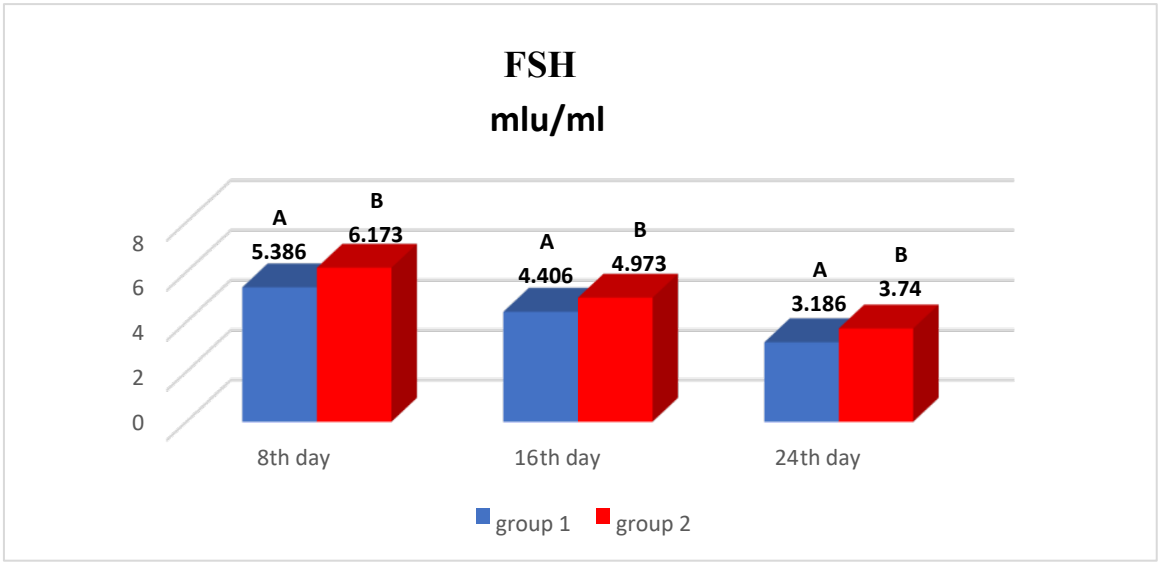


Figure (4.1.2.2): The levels of FSH for similar days in different phases between both groups

4.1.3 LH

First Group:

Results revealed that LH increased significantly ($p \leq 0.05$) in the 16th day (7.073 ± 0.854 mlu/ml) in comparison with both the 8th day (4.506 ± 0.693 mlu/ml) and the 24th day (3.866 ± 0.843 mlu/ml). Table 4.1.1, figure 4.1.3.1.

Second Group:

Results revealed that LH increased significantly ($p \leq 0.05$) in the 16th day (9.140± 0.720 mlu/ml) in comparison with both the 8th day (6.613± 0.966 mlu/ml) and the 24th day (4.086±0.586 mlu/ml). Table 4.1.1, figure 4.1.3.1.

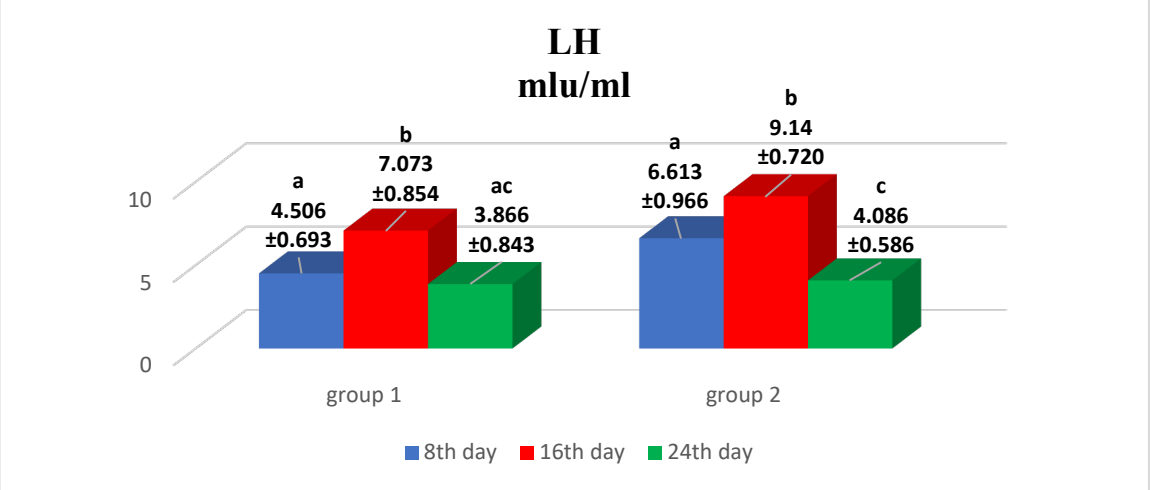


Figure (4.1.3.1): The levels of LH changes during different phases of menstrual cycle in both groups.

Between Groups:

LH increased non-significantly in the second group in comparison with the first group for all similar days. Table 4.1.2, figure 4.1.3.2.

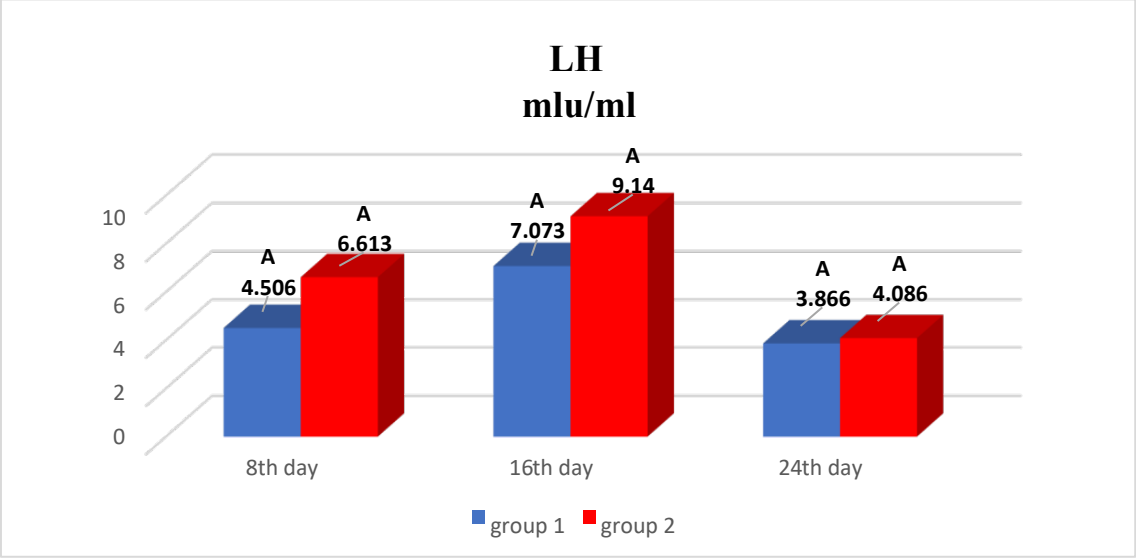


Figure (4.1.3.2): The levels of LH for similar days in different phases between both groups.

4.1.4. Estradiol

First Group:

Results revealed that estradiol increased non-significantly in the 8th day (95.733 ± 8.562 pg/ml) in comparison with the16th day (90.146 ± 4.362 pg/ml) and significantly (p≤0.05) with the 24th day (70.466 ± 5.235 pg/ml). Table 4.1.1, figure 4.1.4.1.

Second Group:

Results revealed that estradiol increased non-significantly in the 8th day (93.733 ± 5.314 pg/ml) in comparison with the16th day (88.933 ± 4.45 pg/ml) and significantly (p≤0.05) with the 24th day (67.866 ± 4.858 pg/ml). Table 4.1.1, figure 4.1.4.1

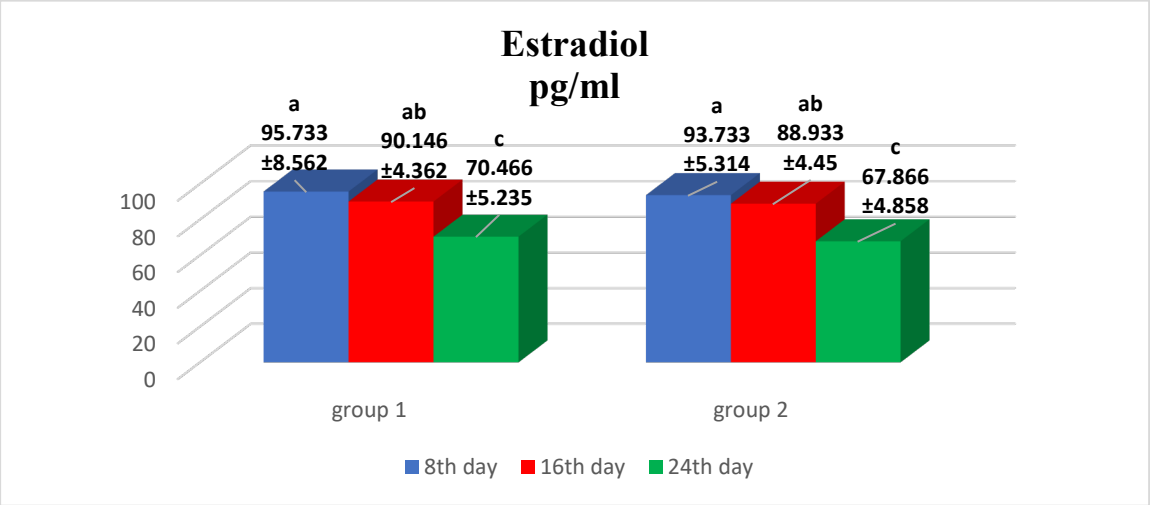


Figure (4.1.4.1): The levels of estradiol changes during different phases of menstrual cycle in both groups.

Between Groups:

Estradiol decreased non-significantly in the second group in comparison with the first group for all similar days. Table 4.1.2, figure 4.1.4.2.

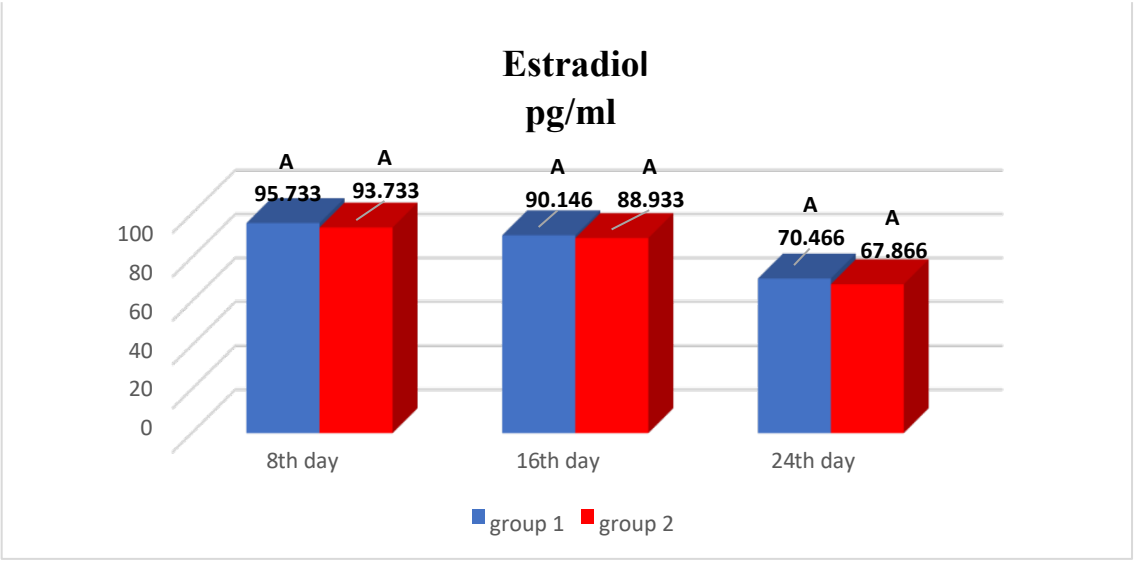


Figure (4.1.4.2): The levels of estradiol for similar days in different phases between both groups.

4.1.5 Progesterone

First Group:

Results revealed that progesterone increased significantly ($p \leq 0.05$) in the 24th day (6 ± 0.985 ng/ml) in comparison with the 8th day (0.960 ± 0.638 ng/ml) and 16th day (2.14 ± 0.358 ng/ml). Table 4.1.1, figure 4.1.5.1.

Second Group:

Results revealed that progesterone increased significantly ($p \leq 0.05$) in the 24th day (5.986 ± 0.988 ng/ml) in comparison with the 8th day (0.786 ± 0.331 ng/ml) and 16th day (1.493 ± 0.29 ng/ml). Table 4.1.1, figure 4.1.5.1.

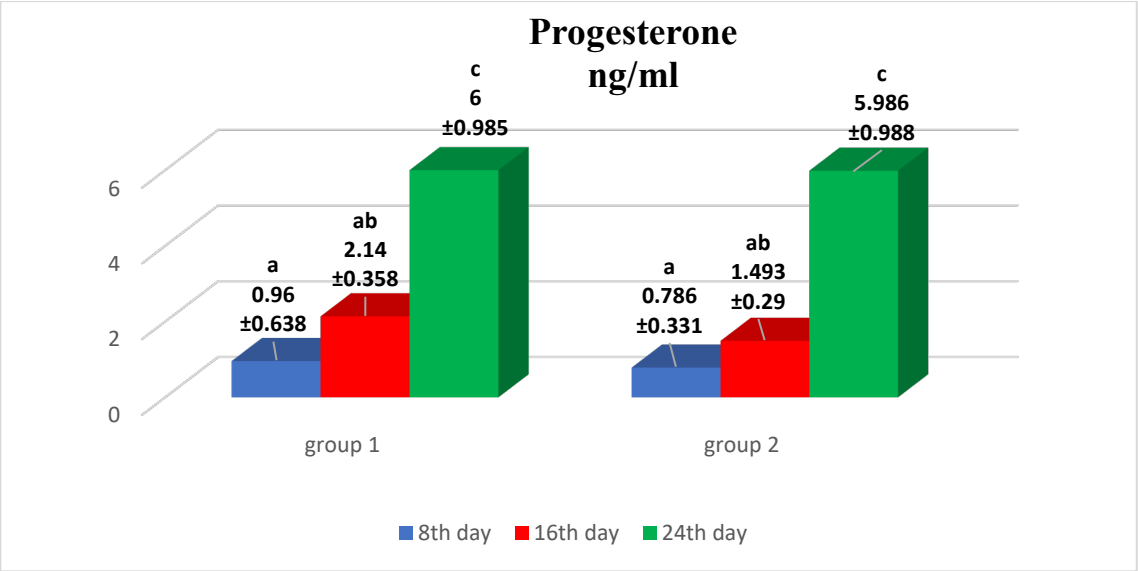


Figure (4.1.5.1): The levels of progesterone changes during different phases of menstrual cycle in both groups.

Between Groups:

Progesterone decreased non-significantly in all days in the second group in comparison with the first group for all similar days. Table 4.1.2, figure 4.1.5.2.

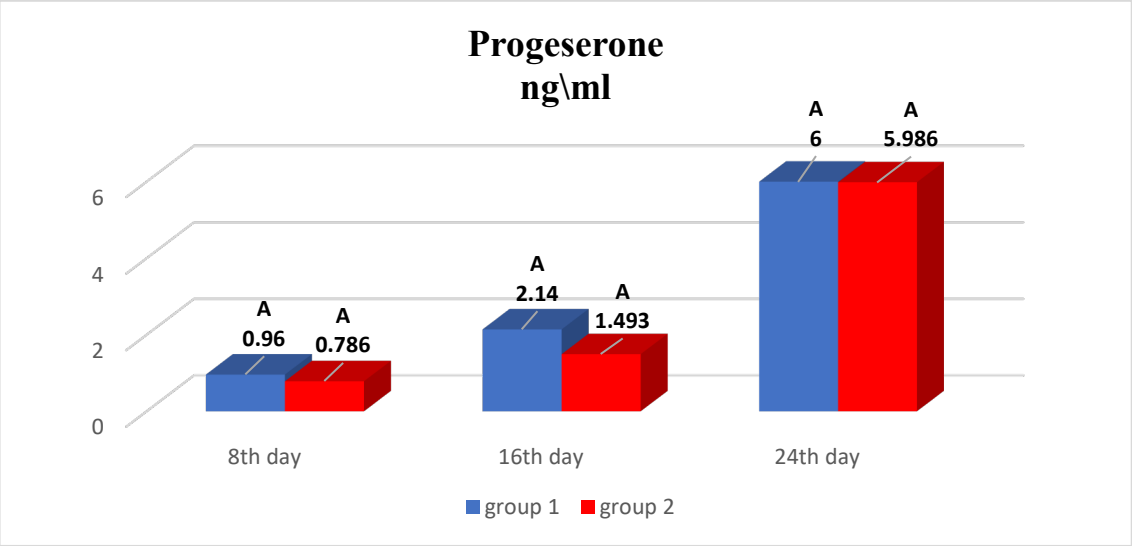


Figure (4.1.5.2): The levels of progesterone for similar days in different phases between both groups.

4.1.6 Prolactin

First Group:

Results revealed that prolactin increased significantly ($p \leq 0.05$) in the 16th day (24.600 ± 5.543 ng/ml)) in comparison with the 8th day (14.860 ± 3.334 ng/ml) and non-significantly with the 24th day (20.200 ± 3.497 ng/ml). Table 4.1.1, figure 4.1.6.1.

Second Group:

Results revealed that prolactin increased significantly ($p \leq 0.05$) in the 16th day (25.326 ± 5.589 ng/ml) in comparison with the 8th day (15.840 ± 3.650 ng/ml) and non-significantly with the 24th day (21.473 ± 3.440 ng/ml). Table 4.1.1, figure 4.1.6.1.

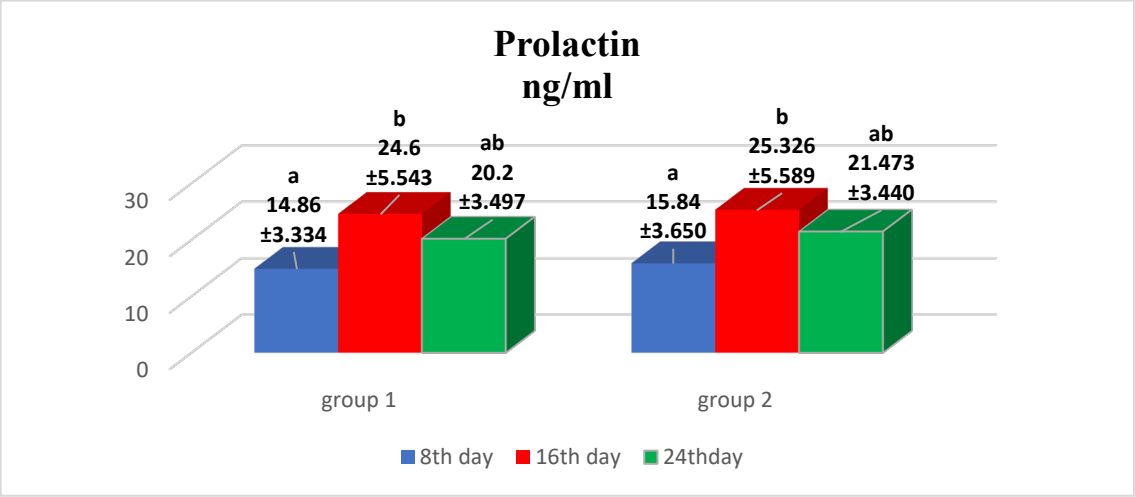


Figure (4.1.6.1): The levels of prolactin changes during different phases of menstrual cycle in both groups.

Between Groups:

Prolactin increased non-significantly in the second group in comparison with the first group for all similar days. Table 4.1.2, figure 4.1.6.2.

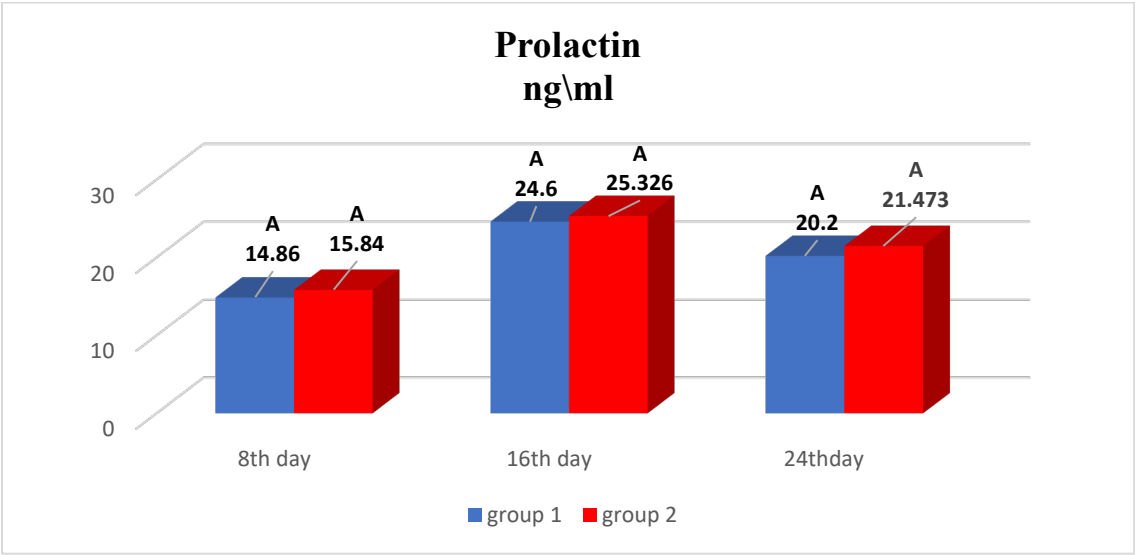


Figure (4.1.6.2): The levels of prolactin for similar days in different phases between both groups.

Table (4.1.1): Hormonal changes during different phases of menstrual cycle in both groups.

Groups / Parameters	Group 1 20-25years			Group 2 40-45 years		
	8th day	16th day	24th day	8th day	16th day	24th day
Kisspeptin pg/ml	a 229.06 ±15.986	ab 274.86 ±19.202	ac 222.4 ±15.767	a 251.666 ±28.192	ab 298.8 ±28.598	ac 239.133 ±27.376
FSH mlu/ml	a 5.386 ±0.883	ab 4.406 ±0.717	bc 3.186 ±0.680	a 6.173 ±0.459	b 4.973 ±0.367	c 3.740 ±0.421
LH mlu/ml	a 4.506 ±0.693	b 7.073 ±0.854	ac 3.866 ±0.843	a 6.613 ±0.966	b 9.140 ±0.720	c 4.086 ±0.586
Estradiol pg/ml	a 95.733 ±8.562	ab 90.146 ±4.362	c 70.466 ±5.235	a 93.733 ±5.314	ab 88.933 ±4.45	c 67.866 ±4.858
Progesterone ng/ml	a 0.960 ±0.638	ab 2.14 ±0.358	c 6 ±0.985	a 0.786 ±0.331	ab 1.493 ±0.29	c 5.986 ±0.988
Prolactin ng/ml	a 14.860 ±3.334	b 24.600 ±5.543	ab 20.200 ±3.497	a 15.840 ±3.650	b 25.326 ±5.589	ab 21.473 ±3.440

- **Values represent means ±SD.**
- **The small different letters represent a significant difference ($p \leq 0.05$) among the different days for each group.**
- **The small similar letters represent no significant differences among the different days for each group.**

Table (4.1.2): Hormonal changes for similar days in different phases between both groups.

Days / Parameters	8th day G1	8th day G2	16th day G1	16th day G2	24th day G1	24th day G2
Kisspeptin pg/ml	A 229.06 ±15.986	B 251.66 ±28.192	A 274.86 ±19.202	B 298.8 ±28.598	A 222.4 ±15.767	B 239.133 ±27.376
FSH mlu/ml	A 5.38 ±0.883	B 6.17 ±0.459	A 4.40 ±0.717	B 4.97 ±0.367	A 3.18 ±0.680	B 3.74 ±0.421
LH mlu/ml	A 4.50 ±0.693	A 6.61 ±0.966	A 7.07 ±0.854	A 9.14 ±0.720	A 3.86 ±0.843	A 4.08 ±0.586
Estradiol pg/ml	A 95.73 ±8.562	A 93.73 ±5.314	A 90.14 ±4.362	A 88.93 ±4.45	A 70.46 ±5.235	A 67.86 ±4.858
Progesterone ng/ml	A 0.96 ±0.638	A 0.78 ±0.331	A 2.14 ±0.358	A 1.49 ±0.29	A 6 ±0.985	A 5.98 ±0.988
Prolactin ng/ml	A 14.86 ±3.334	A 15.84 ±3.650	A 24.60 ±5.543	A 25.32 ±5.589	A 20.20 ±3.497	A 21.47 ±3.440

- **Values represent means.**
- **The capital different letters represent a significant difference ($p \leq 0.05$) among the similar days between groups.**
- **The capital similar letters represent no significant differences among the similar days between groups.**

4.2 Biochemical parameters

4.2.1 H_2O_2

First Group:

Results revealed that H_2O_2 increased non-significantly in the 16th day (5.712 ± 0.843 pg/ml) in comparison with the 8th day (5.613 ± 0.873 pg/ml)) and significantly ($p \leq 0.05$) with the 24th day (4.048 ± 0.729 pg/ml). Table 4.2.1, figure 4.2.1.1.

Second Group:

Results revealed that H_2O_2 increased non-significantly in the 16th day (6.293 ± 0.437 pg/ml) in comparison with the 8th day (6.149 ± 0.527 pg/ml) and significantly ($p \leq 0.05$) with the 24th day (4.567 ± 0.481 pg/ml). Table 4.2.1, figure 4.2.1.1.

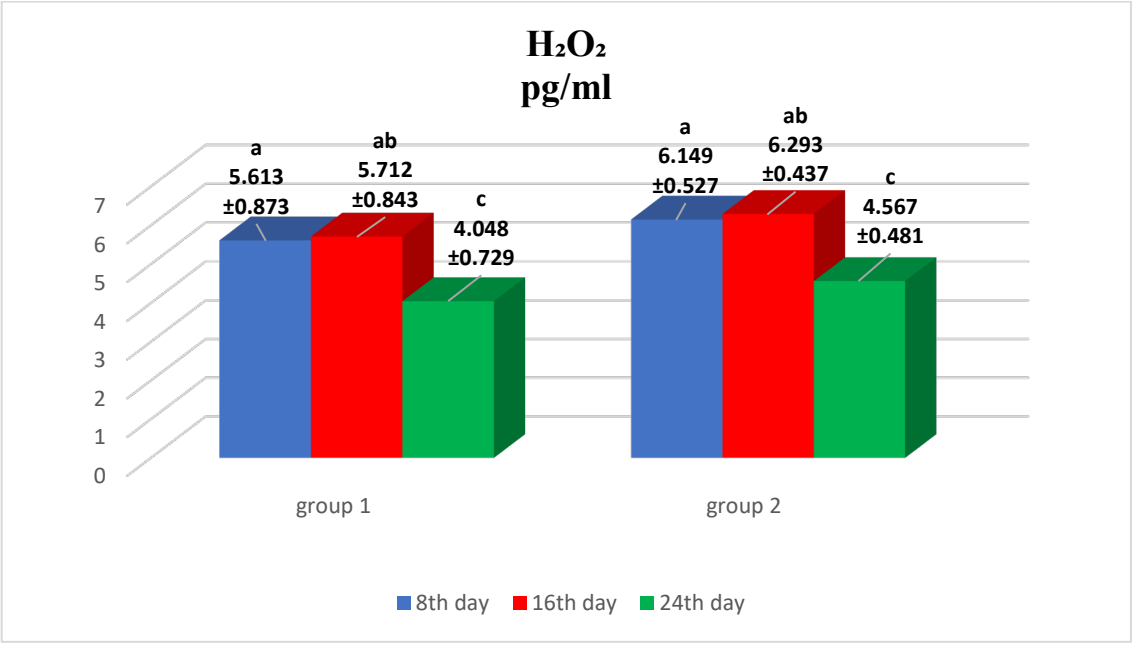


Figure (4.2.1.1): The levels of H_2O_2 changes during different phases of menstrual cycle in both groups.

Between Groups:

H_2O_2 increased significantly ($p \leq 0.05$) in the second group in comparison with the first group for all similar days. Table 4.2.2, figure 4.1.2.2.

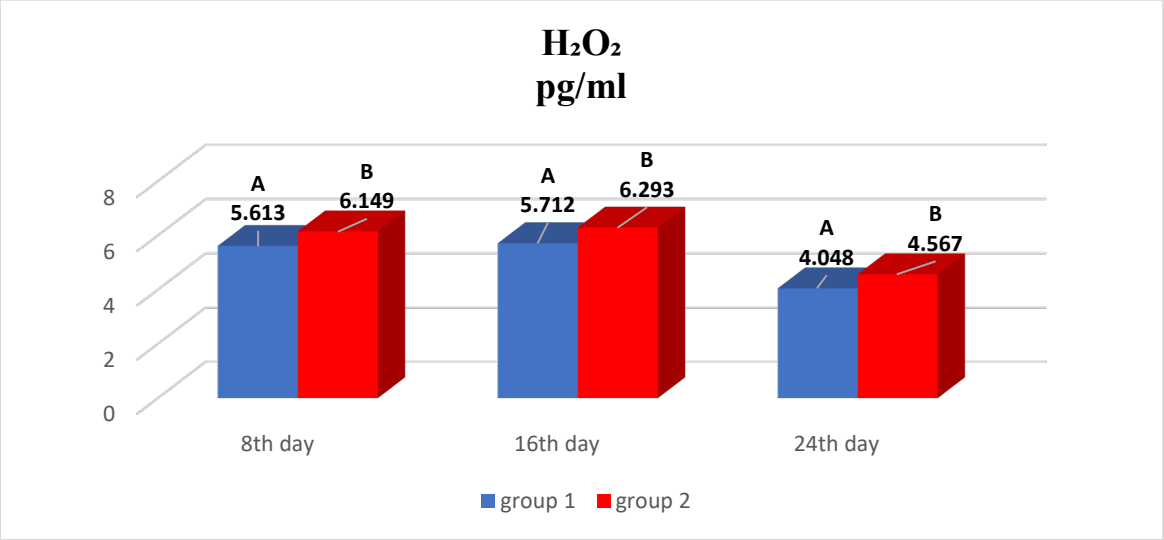


Figure (4.2.1.2): The levels of H_2O_2 for similar days in different phases between both groups.

4.2.2 CAT

First Group:

Results revealed that CAT increased non-significantly in the 8th day (51.280 ± 6.369 KU/L) in comparison with the 16th day (48.038 ± 6.223 KU/L) and significantly ($p \leq 0.05$) with the 24th day (38.582 ± 7.360 KU/L). Table 4.2.1, figure 4.2.2.1.

Second Group:

Results revealed that CAT increased non-significantly in the 8th day (43.033 ± 4.538 KU/L) in comparison with the 16th day (42.740 ± 4.161 KU/L) and significantly ($p \leq 0.05$) with the 24th day (33.340 ± 3.334 KU/L). Table 4.2.1, figure 4.2.2.1.

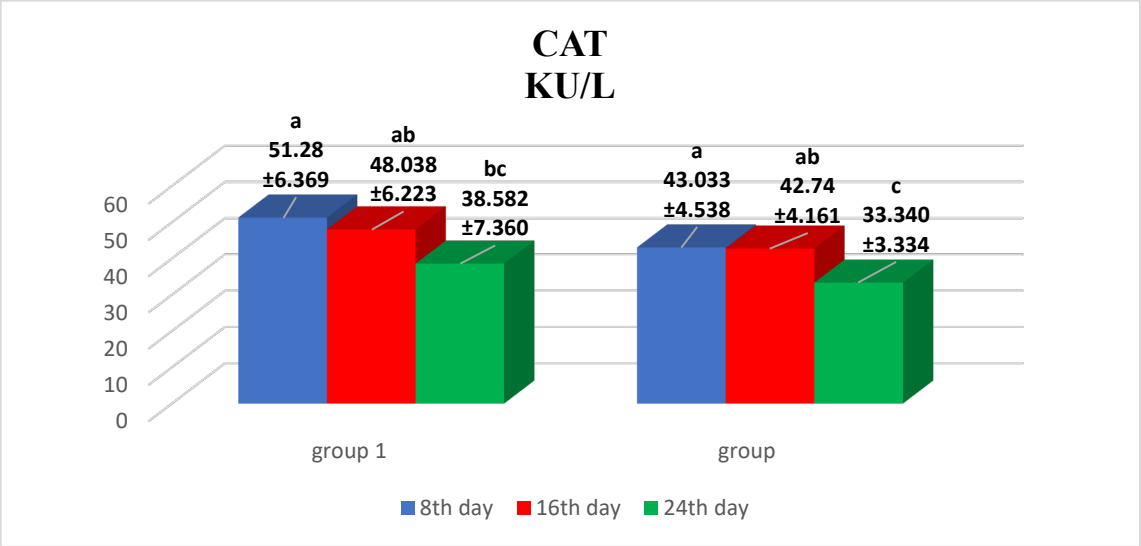


Figure (4.2.2.1): The levels of CAT changes during different phases of menstrual cycle in both groups.

Between Groups:

CAT decreased significantly ($p \leq 0.05$) in all days in second group in comparison with first group for all similar days. Table 4.2.2, figure 4.2.2.2.

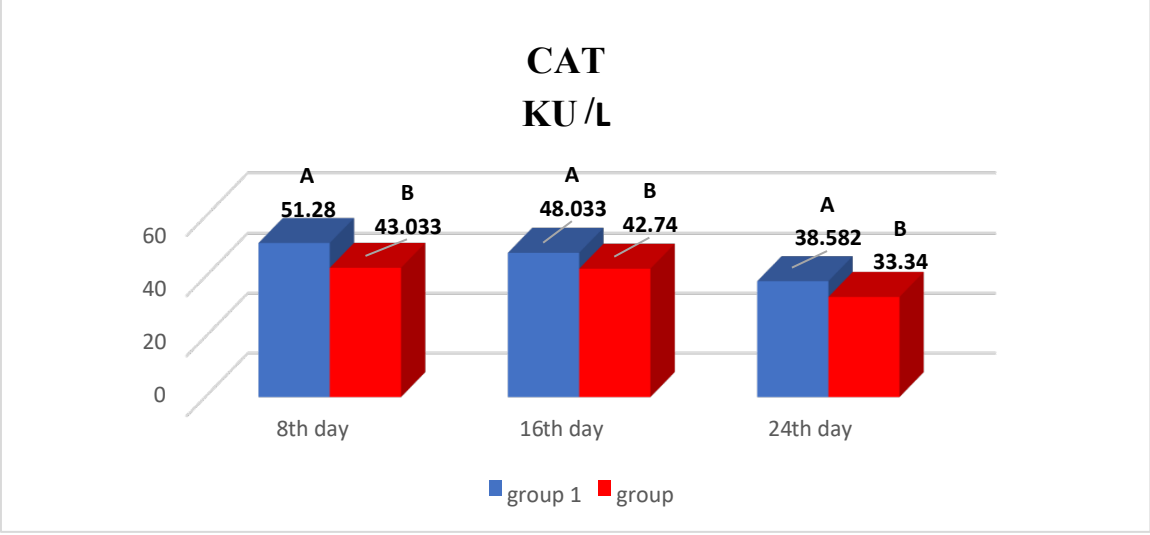


Figure (4.2.2.2): The levels of CAT for similar days in different phases between both groups.

4.2.3 LCAT

First Group:

Results revealed that LCAT increased non-significantly in the 8th day (98.026 ± 6.424 U/L) in comparison with the 16th day (91.586 ± 5.807 U/L) and significantly ($p \leq 0.05$) with the 24th day (86.746 ± 5.676 U/L). Table 4.2.1, figure 4.2.3.1.

Second Group:

Results revealed that LCAT increased non-significantly in the8th day (84.674 ± 5.722 U/L) in comparison with the16th day (79.835 ± 5.434 U/L) and significantly ($p \leq 0.05$) with the 24th day (68.194 ± 4.326 U/L). Table 4.2.1, figure 4.2.3.1.

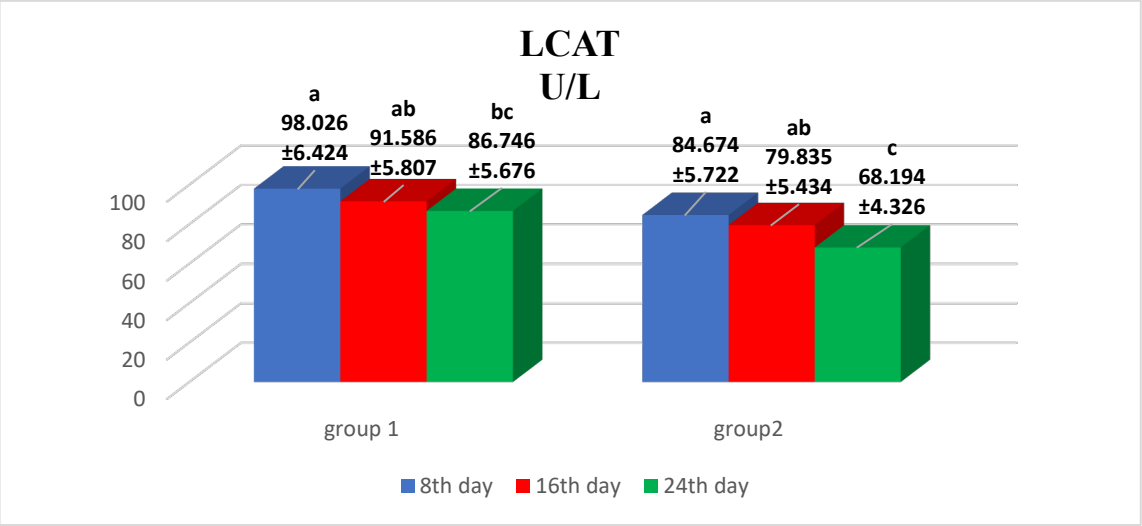


Figure (4.2.3.1): The levels of LCAT changes during different phases of menstrual cycle in both groups.

Between Groups:

LCAT decreased non-significantly in the second group in comparison with the first group for all similar days. Table 4.2.2, figure 4.2.3.2.

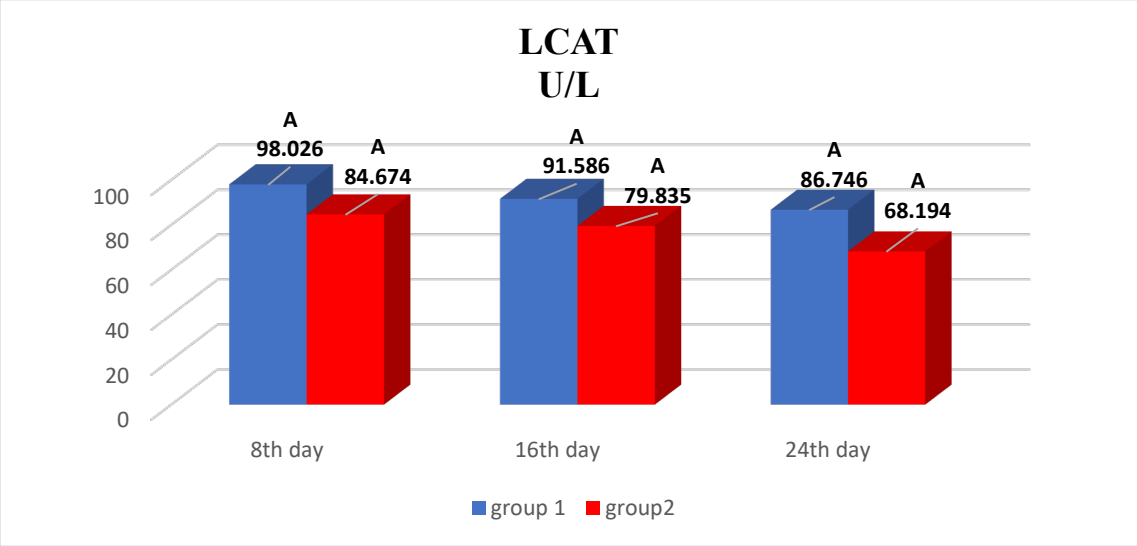


Figure (4.2.3.2): The levels of LCAT for similar days in different phases between both groups.

4.2.4 G-CSF

First Group:

Results revealed that G-CSF increased non-significantly in the 16th day (143.733 ± 6.128 pg/ml) in comparison with the 8th day (141.266 ± 8.027 pg/ml) and significantly ($p \leq 0.05$) with the 24th day (118 ± 6.412pg/ml). Table 4.2.1, figure 4.2.4.1.

Second Group:

Results revealed that G-CSF increased non-significantly in the 16th day (150.266 ± 7.335 pg/ml) in comparison with the 8th day (148.133 ± 7.663 pg/ml) and significantly ($p \leq 0.05$) with the 24th day (124.066 ± 7.845 pg/ml). Table 4.2.1, figure 4.2.4.1.

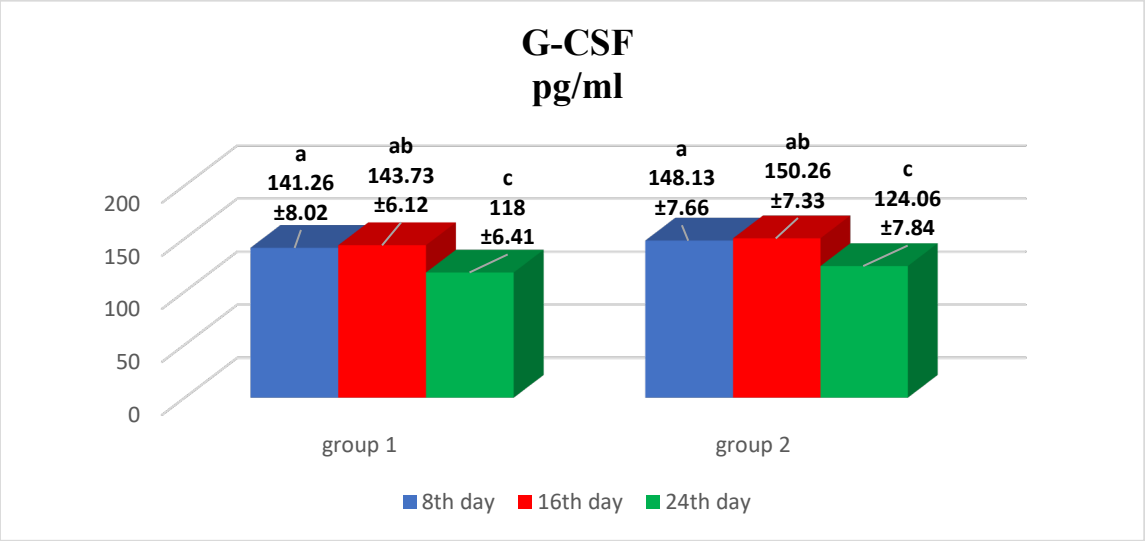


Figure (4.2.4.1): The levels of G-CSF changes during different phases of menstrual cycle in both groups.

Between Groups:

G-CSF increased significantly ($p \leq 0.05$) in the second group in comparison with the first group for all similar days. Table 4.2.2, figure 4.2.4.2.

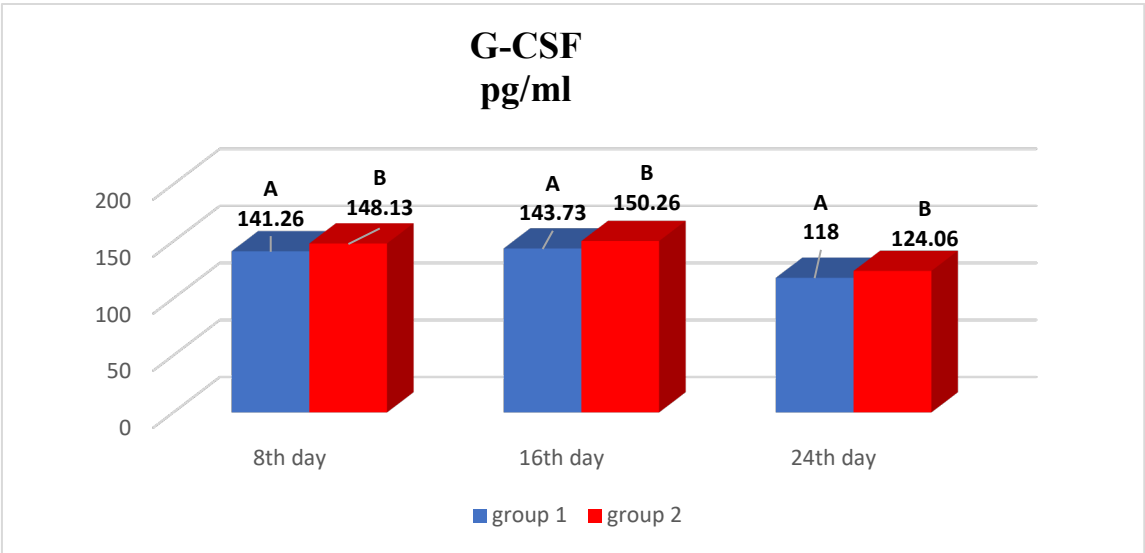


Figure (4.2.4.2): The levels of G-CSF for similar days in different phases between both groups.

4.2.5 WBC

First Group:

Results revealed that WBC increased non-significantly in the 16th day (6.826± 0.659 $10^3/mm^3$) in comparison with the 8th day (6.613± 0.678 $10^3/mm^3$) and significantly ($p \leq 0.05$) with the 24th day (4.96 ± 0.493 $10^3/mm^3$). Table 4.2.1, figure 4.2.5.1.

Second Group:

Results revealed that WBC increased non-significantly in the16th day (7.326 ± 0.559 $10^3/mm^3$) in comparison with the 8th day (7.146 ± 0.540 $10^3/mm^3$) and significantly ($p \leq 0.05$) with the 24th day (5.426 ± 0.533 $10^3/mm^3$). Table 4.2.1, figure 4.2.5.1.

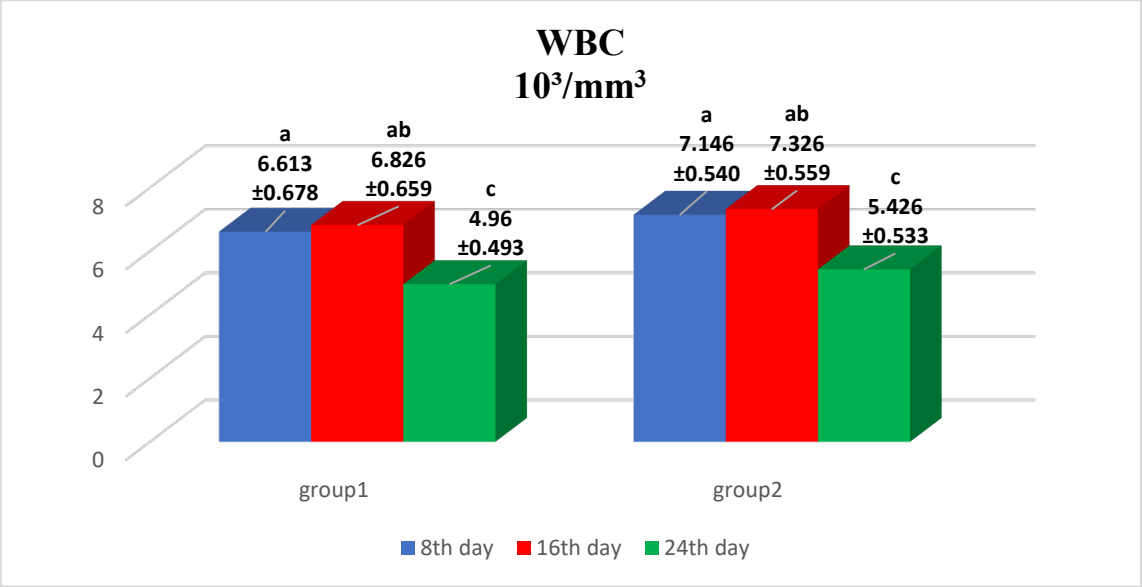


Figure (4.2.5.1): The levels of WBC changes during different phases of menstrual cycle in both groups.

Between Groups:

WBC increased significantly ($p \leq 0.05$) in the second group in comparison with the first group for all similar days. Table 4.2.2, figure 4.2.5.2.

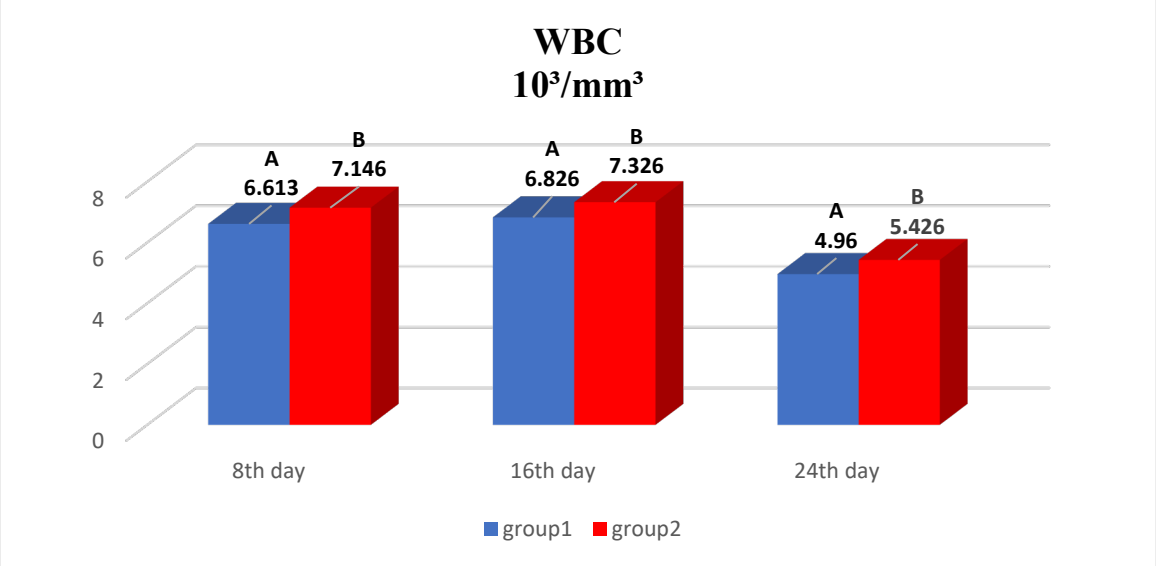


Figure (4.2.5.2): The levels of WBC for similar days in different phases between both groups.

Table (4.2.1): Biochemical changes during different phases of menstrual cycle in both groups.

Groups / Parameters	Group 1 20-25 years			Group2 40-45 years		
	8th day	16th day	24th day	8th day	16th day	24thday
H_2O_2 pg/ml	a 5.613 ±0.873	ab 5.712 ±0.843	c 4.048 ±0.729	a 6.149 ±0.527	ab 6.293 ±0.437	c 4.567 ±0.481
CAT KU/L	a 51.280 ±6.369	ab 48.038 ±6.223	bc 38.582 ±7.360	a 43.033 ±4.538	ab 42.740 ±4.161	c 33.340 ±3.334
LCAT U/L	a 98.026 ±6.424	ab 91.586 ±5.807	bc 86.746 ±5.676	a 84.674 ±5.722	ab 79.835 ±5.434	c 68.194 ±4.326
G-SCF pg/ml	a 141.266 ±8.027	ab 143.733 ±6.128	c 118 ±6.412	a 148.133 ±7.663	ab 150.266 ±7.335	c 124.06 ±7.845
WBC $10^3/mm^3$	a 6.613 ±0.678	ab 6.826 ±0.659	c 4.96 ±0.493	a 7.146 ±0.540	ab 7.326 ±0.559	c 5.426 ±0.533

- **Values represent means ±SD.**
- **The small different letters represent a significant difference ($p \leq 0.05$) among the different days for each group.**
- **The small similar letters represent no significant differences among the different days for each group.**

Table (4.2.2): Biochemical changes for similar days in different phases between both groups.

Days / Parameters	8th day G1	8th day G2	16th day G1	16thday G2	24th day G1	24th day G2
H_2O_2 pg/ml	A 5.61 ±0.873	B 6.149 ±0.527	A 5.71 ±0.843	B 6.29 ±0.437	A 4.04 ±0.729	B 4.56 ±0.481
CAT KU/L	A 51.28 ±6.369	B 43.03 ±4.538	A 48.03 ±6.223	B 42.74 ±4.161	A 38.58 ±7.360	B 33.34 ±3.334
LCAT U/L	A 98.02 ±6.424	A 84.67 ±5.722	A 91.58 ±5.807	A 79.83 ±5.434	A 86.74 ±5.676	A 68.19 ±4.326
G-CSF pg/ml	A 141.26 ±8.027	B 148.13 ±7.663	A 143.73 ±6.128	B 150.26 ±7.335	A 118 ±6.412	B 124.06 ±7.845
WBC $10^3/mm^3$	A 6.613 ±0.678	B 7.146 ±0.540	A 6.826 ±0.659	B 7.326 ±0.559	A 4.96 ±0.493	B 5.42 ±0.533

- **Values represent means.**
- **The capital different letters represent a significant difference ($p \leq 0.05$) among the similar days between groups.**
- **The capital similar letters represent no significant differences among the similar days between groups.**

4.3 Lipid profile

4.3.1 TC

First Group:

Results revealed that TC increased non-significantly in the 8th day (161.733 ± 9.749 mg/dl) in comparison with the 16th day (157.266 ± 9.343 mg/dl) and significantly ($p \leq 0.05$) with the 24th day (133.133 ± 7.016 mg/dl) Table 4.3.1, figure 4.3.1.1.

Second Group:

Results revealed that TC increased non-significantly in the 8th day (208.066 ± 10.015 mg/dl) in comparison with the16th day (200.800 ± 8.079 mg/dl) and significantly ($p \leq 0.05$) with the 24th day (190.933 ± 7.428 mg/dl) . Table 4.3.1, figure 4.3.1.1

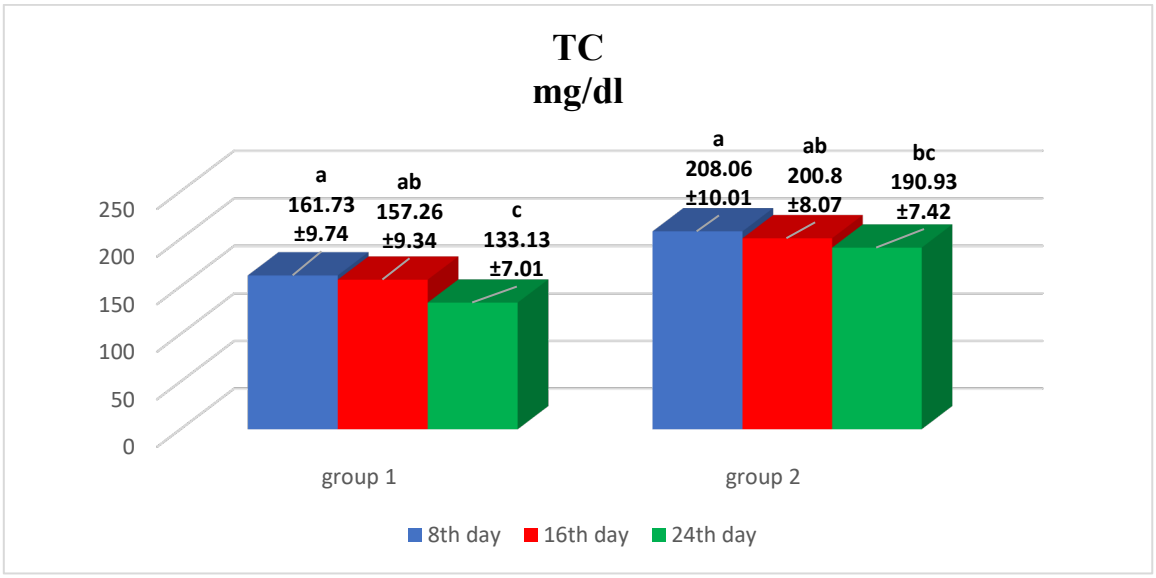


Figure (4.3.1.1): The levels of TC changes during different phases of menstrual cycle in both groups.

Between Groups:

TC increased non-significantly in the second group in compared with the first group for all similar days. Table 4.3.2, figure 4.3.1.2.

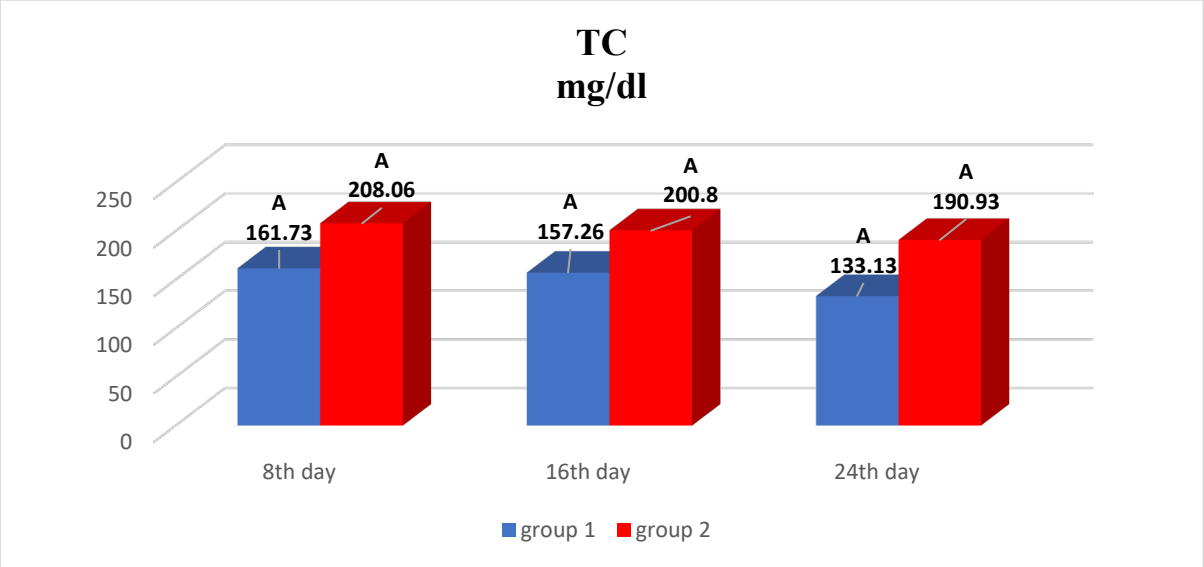


Figure (4.3.1.2): The levels of TC for similar days in different phases between both groups.

4.3.2 TG

First Group:

Results revealed that TG increased non-significantly in the 8th day (99.333 ± 4.932 mg/dl) in comparison with the 16th day (95 ± 4.886 mg/dl) and significantly ($p \leq 0.05$) with the 24th day (88.066 ± 4.575 mg/dl). Table 4.3.1, figure 4.3.2.1.

Second Group:

Results revealed that TG increased non-significantly in the 8th day (157 ± 9.714 mg/dl)) in comparison with the16th day (149.266 ± 9.222 mg/dl) and significantly ($p \leq 0.05$) with the 24th day (130.533 ± 8.053 mg/dl).Table 4.3.1 ،figure 4.3.2.1.

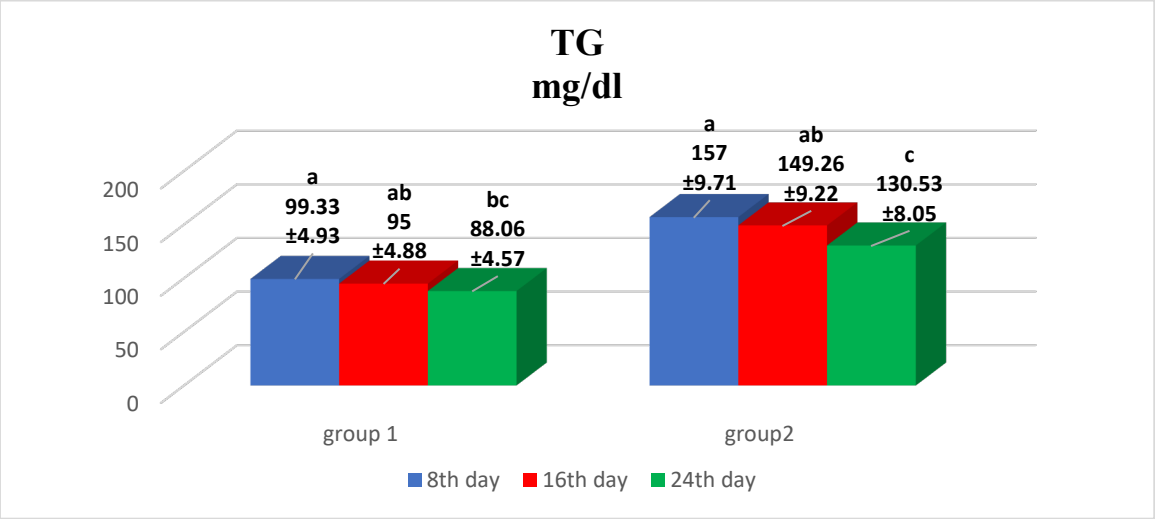


Figure (4.3.2.1): The levels of TG during different phases of menstrual cycle in both groups.

Between Groups:

TG increased non-significantly in the second group in comparison with the first group for all similar days. Table 4.3.2, figure 4.3.2.2.

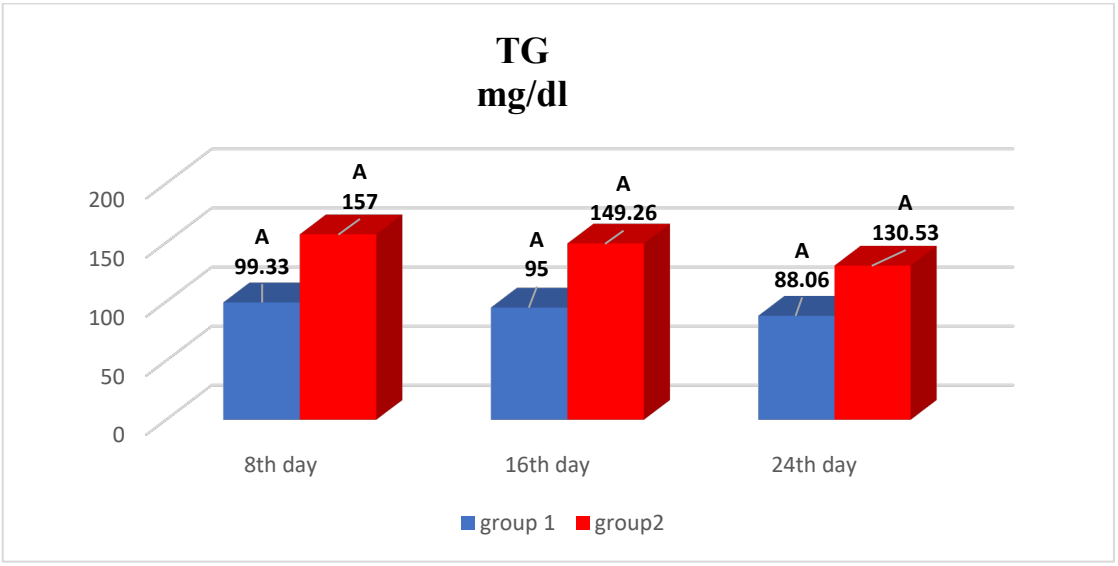


Figure (4.3.2.2): The levels of TG for similar days in different phases between both groups.

4.3.3 HDL

First Group:

Results revealed that HDL increased significantly ($p \leq 0.05$) in the 16th day (64.20 ± 3.940 mg/dl) in comparison with the 8th day (57.266 ± 3.864 mg/dl) and 24th day (52.266 ± 3.815 mg/dl). Table 4.3.1, figure 4.3.3.1.

Second Group:

Results revealed that HDL increased significantly ($p \leq 0.05$) in the 16th day (59.266 ± 3.665 mg /dl) in comparison with the 8th day (52.2±3.892 mg\dl) and 24th day (49.933 ± 3.864 mg/dl). Table 4.3.1, figure 4.3.3.1.

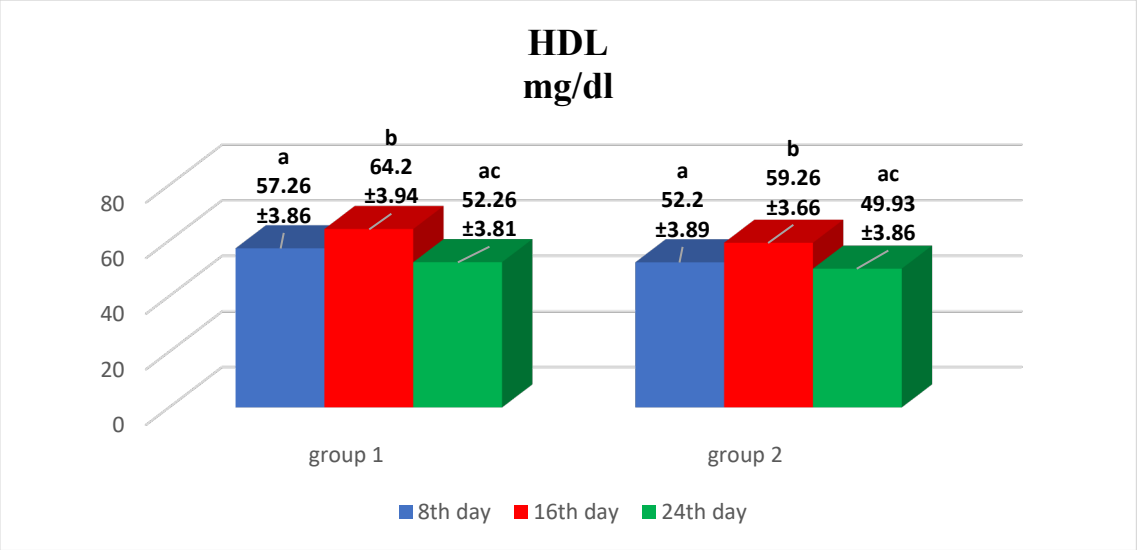


Figure (4.3.3.1): The levels of HDL changes during different phases of menstrual cycle in both groups.

Between Groups:

HDL decreased significantly ($p \leq 0.05$) in both 8th and 16th days in the second group in comparison with the first group. And non-significantly in the 24th day between first and sconed groups. Table 4.3.2, figure 4.3.3.2.

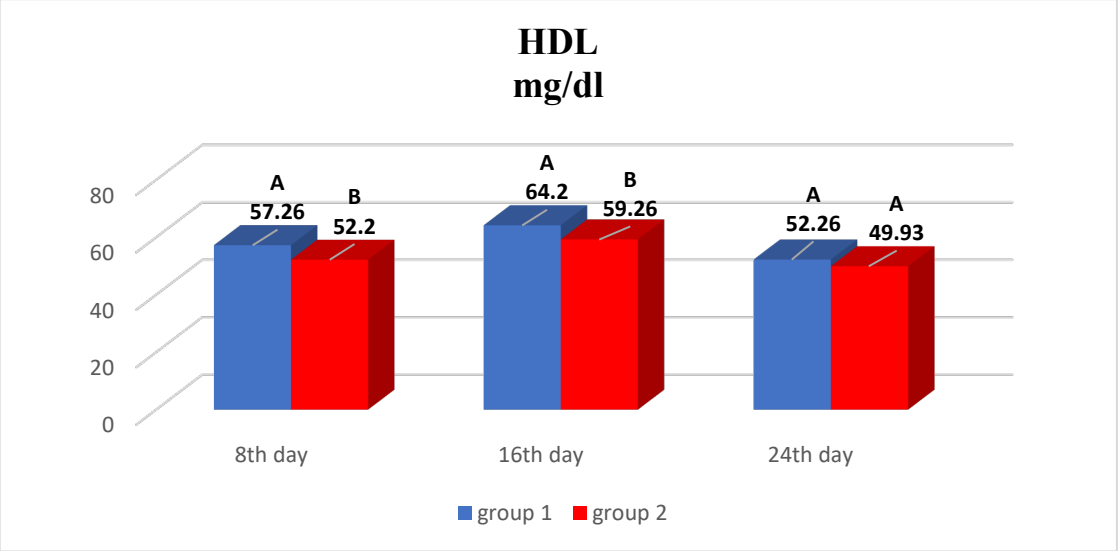


Figure (4.3.3.2): The levels of HDL for similar days in different phases between both groups.

4.3.4 LDL

First Group:

Results revealed that LDL increased significantly ($p \leq 0.05$) in the 8th day (96.733 ± 3.799 mg/dl) in comparison with the 16th day (82.733 ± 4.683 mg/dl) and 24th day (76.333 ± 3.651 mg/dl). Table 4.3.1, figure 4.3.4.1.

Second Group:

Results revealed that LDL increased significantly ($p \leq 0.05$) in the 8th day (121.933 ± 5.584 mg/dl) in comparison with the 16th day (110.866 ± 5.072 mg/dl) and 24th day (105.666 ± 4.856 mg/dl). Table 4.3.1, figure 4.3.4.1.

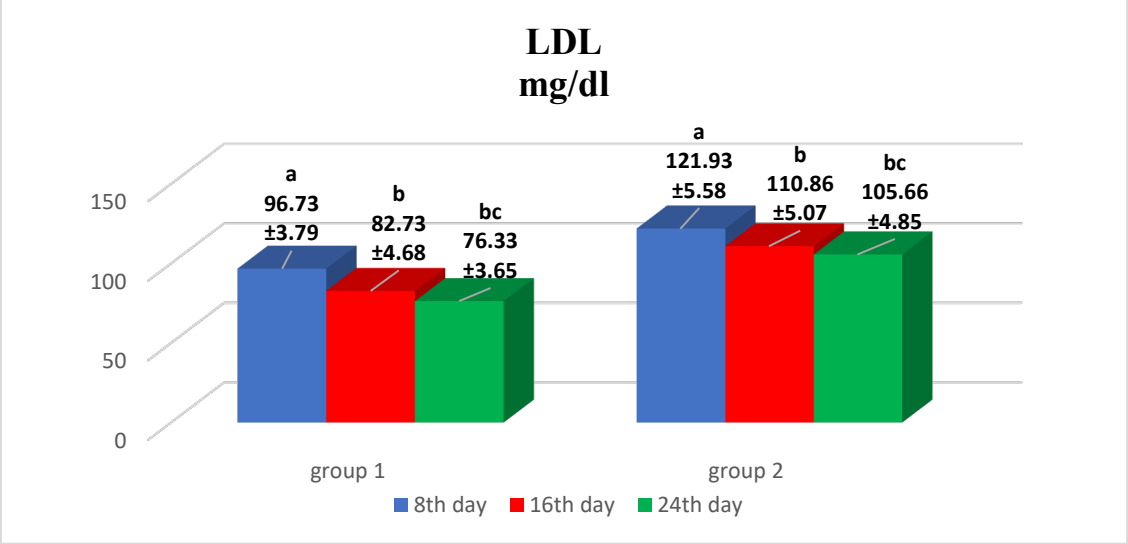


Figure (4.3.4.1): The levels of LDL changes during different phases of menstrual cycle in both groups.

Between Groups:

LDL increased non-significantly in the second group in comparison with the first group for all similar days. Table 4.3.2, figure 4.3.4.2.

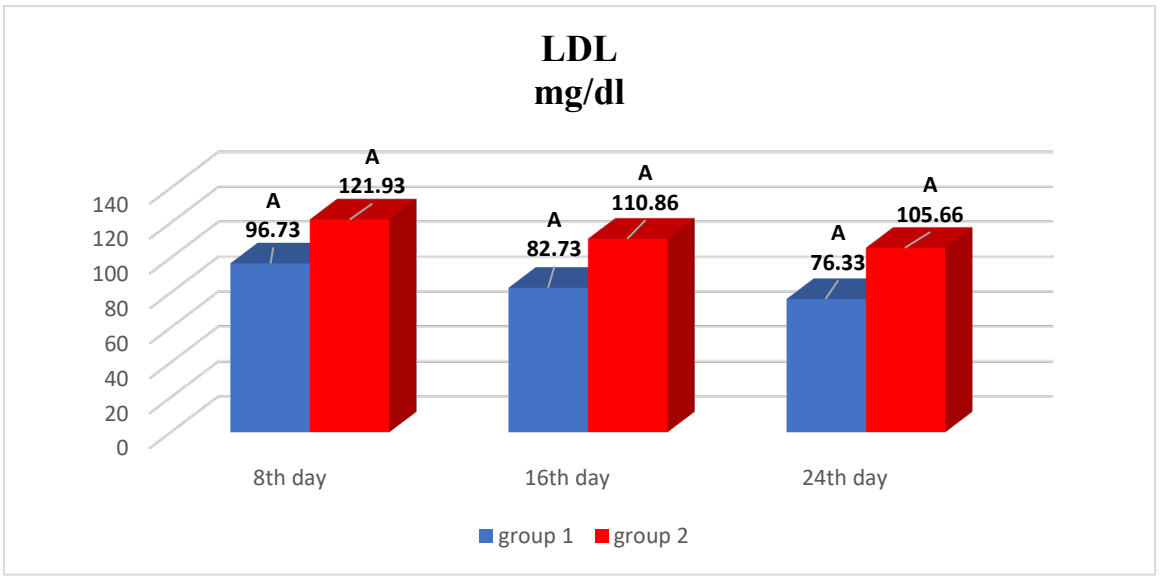


Figure (4.3.4.2): The levels of LDL for similar days in different phases between both groups.

4.3.5 VLDL

First Group:

Results revealed that VLDL increased non-significantly in the 16th day (20.06 ± 3.24 mg/dl) in comparison with the 8th day (18.8 ± 3.06 mg/dl) and 24th day (17.6 ± 3.93 mg/dl). Table 4.3.1, figure 4.3.5.1.

Second Group:

Results revealed that VLDL increased non-significantly in the 16th day (30.13 ± 5.56 mg/dl) in comparison with the 8th day (29.33 ± 5.69 mg\dl) and 24th day (26.33 ± 4.72 mg/dl). Table 4.3.1, figure 4.3.5.1

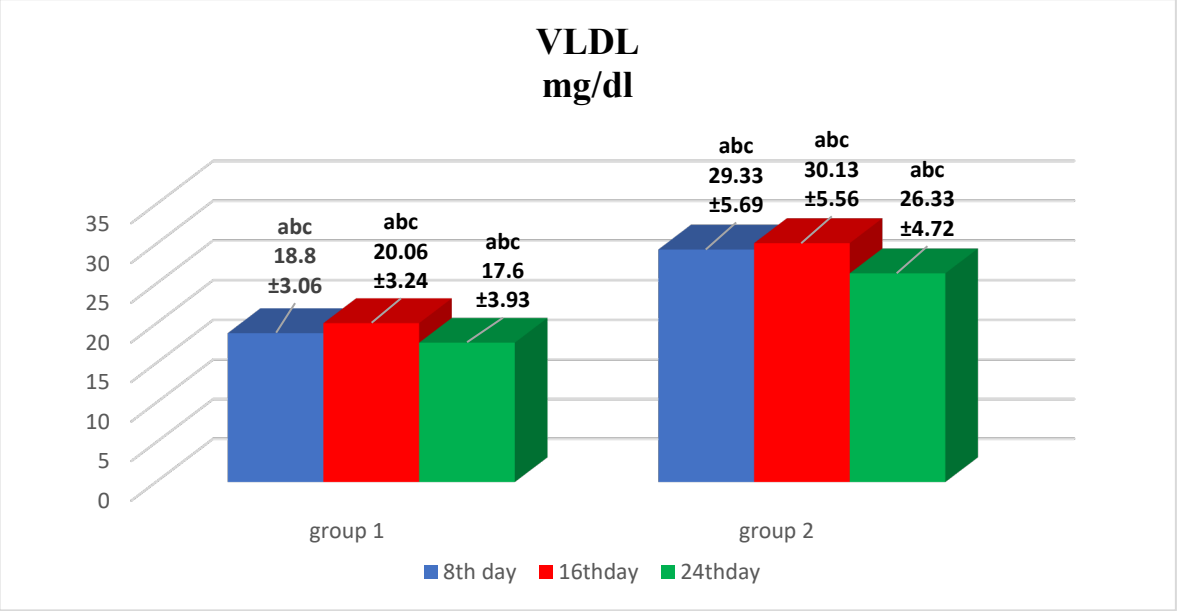


Figure (4.3.5.1): The levels of VLDL changes during different phases of menstrual cycle in both groups.

Between Groups:

VLDL increased non-significantly in the second group in comparison with the first group for all similar days. Table 4.3.2, figure 4.3.5.2.

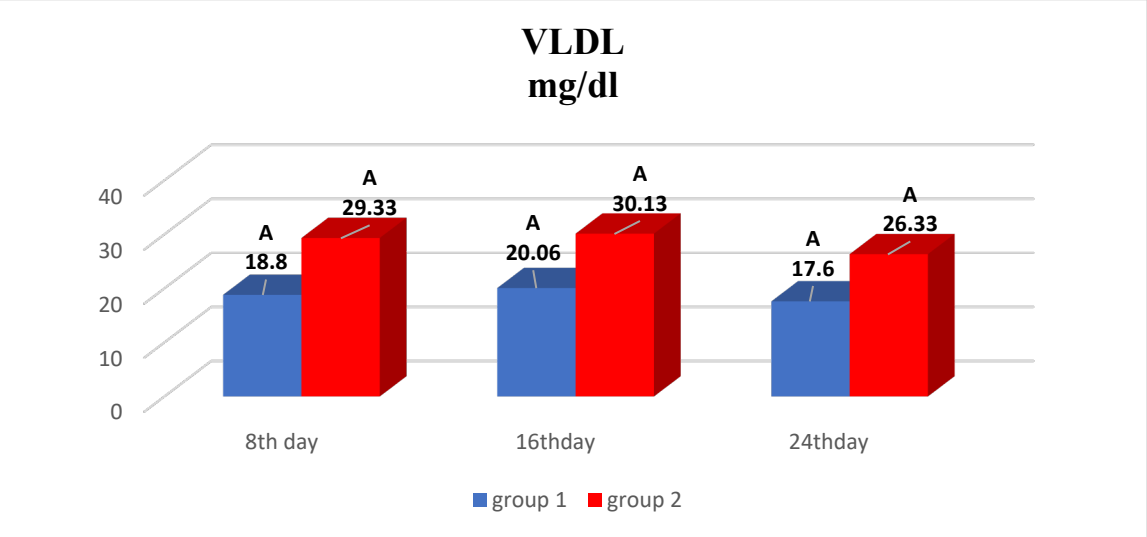


Figure (4.3.5.2): The levels of VLDL for similar days in different phases between both groups.

Table (4.3.1): Lipid profile changes during different phases of menstrual cycle in both groups.

Groups / Parameters	Group 1 20-25years 8th day	Group 1 20-25years 16th day	Group 1 20-25years 24th day	Group 2 40-45years 8th day	Group 2 40-45years 16th day	Group 2 40-45years 24th day
TC mg/dl	a 161.733 ±9.749	ab 157.266 ±9.343	c 133.133 ±7.016	a 208.066 ±10.015	ab 200.8 ±8.079	bc 190.933 ±7.428
TG mg/dl	a 99.333 ±4.932	ab 95 ±4.886	bc 88.066 ±4.575	a 157 ±9.714	ab 149.266 ±9.222	c 130.533 ±8.053
HDL mg/dl	a 57.266 ±3.864	b 64.2 ±3.94	ac 52.26 ±3.815	a 52.2 ±3.892	b 59.26 ±3.665	ac 49.933 ±3.864
LDL mg\dl	a 96.733 ±3.799	b 82.733 ±4.683	bc 76.333 ±3.651	a 121.933 ±5.584	b 110.866 ±5.072	bc 105.666 ±4.856
VLDL mg/dl	abc 18.8 ±3.06	abc 20.06 ±3.24	abc 17.6 ±3.93	abc 29.33 ±5.69	abc 30.13 ±5.56	abc 26.33 ±4.72

- **Values represent means ±SD.**
- **The small different letters represent a significant difference ($p \leq 0.05$) among the different days for each group.**
- **The small similar letters represent no significant differences among the different days for each group.**

Table (4.3.2): Lipid profile change for similar days in different phases between both groups.

Days / Parameters	8th day G1	8th day G2	16th day G1	16th day G2	24th day G1	24th day G2
TC mg/dl	A 161.73 ±9.749	A 208.06 ±10.015	A 157.26 ±9.343	A 200.8 ±8.079	A 133.13 ±7.016	A 190.93 ±7.428
TG mg/dl	A 99.33 ±4.932	A 157 ±9.714	A 95 ±4.8	A 149.26 ±9.222	A 88.06 ±4.575	A 130.53 ±8.053
HDL mg/dl	A 57.26 ±3.864	B 52.20 ±3.892	A 64.20 ±3.94	B 59.26 ±3.665	A 52.26 ±3.815	A 49.93 ±3.864
LDL mg/dl	A 96.73 ±3.799	A 121.93 ±5.584	A 82.73 ±4.683	A 110.86 ±5.072	A 76.33 ±3.651	A 105.66 ±4.856
VLDL mg/dl	A 18.8 ±3.06	A 29.33 ±5.69	A 20.06 ±3.24	A 30.13 ±5.56	A 17.6 ±3.93	A 26.33 ±4.72

- **Values represent means.**
- **The capital different letters represent a significant difference ($p \leq 0.05$) among the similar days between groups.**
- **The capital similar letters represent no significant differences among the similar days between groups.**

Chapter Five

Discussion

5.1 Kisspeptin

The current results revealed that kisspeptin levels increased significantly ($p \leq 0.05$) in the 16th day in comparison with other days in both groups (Table 4.1.1, figure 4.1.1.1)

This elevation may be indicated the possible role of kisspeptin in ovulatory process due to its impacts on upregulates GnRH secretion and as a key component of the LH surge, especially, the present LH levels increased in 16th day (Table 4.1.1, figure 4.3.1.1), in addition, ovarian steroid (estradiol and progesterone) may be exert a positive and or a negative feedback actions on the kisspeptin secretion sites , whereas, the current estradiol and progesterone hormones decreased and increased significantly in the same day of the cycle (16th day), respectively. (Table 4.1.1, figure 4.1.4.1, figure 4.1.5.1).

These results and ideas are in agreements with following studies. Kisspeptin a neuropeptide that upregulates GnRH secretion and considered as an essential element for the LH surge and ovulation, whereas, kisspeptin expression increases just before ovulation and LH surge (Smith *et al*.,2006; Dungan *et al*.,2007; Ibrahim *et al*.,2020).

Furthermore, Barabas and his colleagues 2020 found that during the menstrual cycle, kisspeptin is upstream GnRH which produced by the hypothalamus via GnRH neurons directly influence the secretion of FSH and LH from the anterior pituitary gland, whereas, the LH surge that occurs at the mid-cycle is responsible for the start of ovulation, hence, kisspeptin is a fundamental component in creating LH surge and ovulation (Trevisan *et al*.,2018; Aquino *et al*.,2019).

Furthermore, estrogen exert a positive and negative feedback mechanism on kisspeptinergic neurons via ER α to secrete kisspeptin at the times of LH surge and at the end of the follicular phase (FSH decrease), respectively (Hameed *et al*.,2011).

Moreover, Mishra and his colleagues 2019 showed that kisspeptin, neurokinin-B and dynorphin neurons regulate the surge and pulsatile centers of GnRH in the hypothalamus and are modulated by the ovarian steroids.

In addition, the ovarian steroids estradiol and progesterone exert critical suppressive and stimulatory actions upon the brain to control GnRH release that drives menstrual cycle (Herbison, 2020).

Furthermore, estrogen levels increase abruptly just before ovulation to cause LH surge that triggers ovulation via positive feedback to GnRH neurons, but this neuron does not express the estrogen receptor, while, kisspeptin neuron express this receptor (Clarkson *et al*.,2009). Studies demonstrated that progesterone also involved in the positive feedback and LH surge, whereas, kisspeptin neurons express progesterone receptor (Gal *et al*., 2016; Mittelmam-smith *et al.,*2017).

In addition, Foradori and his colleagues 2005 showed that progesterone involved in kisspeptin regulation and it has been demonstrated that progesterone receptor co-localized in KNDy neurons in the hypothalamus.

On the other hand, the present results revealed that kisspeptin levels increased in all days significantly ($p \leq 0.05$) (except the 24^{th} day) of the second group in comparison with the similar days of the first group (Table 4.1.2, figure 4.1.1.2).

The current deficiency of estradiol and progesterone levels associated with progressive age (second group), (Table 4.1.2, figure 4.1.4.2, figure 4.1.5.2), may

be beyond the high levels of kisspeptin in this group by their positive and negative feedback mechanism on the kisspeptin production, that leading to high levels of GnRH secretion and more gonadotropins, as the present results pointed out a high level of FSH and LH in second group (Table 4.1.2, figure 4.1.2.2, figure 4.1.3.2). Furthermore, premenopausal women that second group included are characterized with high and low levels of gonadotropins and sex steroid hormones, respectively (Table 4.1.2, figure 4.1.2.2, figure 4.1.3.2).

These results and findings are in agreement with the observations of many studies. There is an initial decrease in ovarian steroids and an increase in kisspeptin levels during progressive age, this increase may occur due to the lack of estrogen negative feedback (Hall,2007; Hrabovszky *et al*.,2019; Ibrahim,2020).

In addition, Ibrahim and his colleagues 2020 showed that kisspeptin serum levels increase with age, whereas, its higher in women above 35 years than those less 24 years, whereas, alteration in estrogen level at late and end reproductive age may participate in elevating the kisspeptin level.

5.2 follicle stimulating hormone (FSH)

The present results appeared an increase tendency in FSH level at the 8^{th} day of the cycle for the first and the second groups (Table 4.1.1, figure 4.1.2.1).

This increase may be explained by the influence of the ovarian steroids feedback (positive and or negative) mechanism on the hypothalamus and or anterior pituitary and a subsequent release of GnRH and FSH, particularly, the current findings indicated that estradiol and progesterone hormones increased and decreased respectively at the 8th day in comparison with other days in both groups (Table 4.1.1, figure 4.1.4.1, figure 4.1.5.1).

These results and findings are in agreement with the observations of many studies. The ovarian steroids are the principal mediators of the stimulating or suppressing effect on gonadotropin secretion during the menstrual cycle via a positive and negative feedback mechanism (Messinis, 2006; Gal *et al.*,2016).

In addition , the ovarian steroids exert critical stimulatory and suppressive actions upon the brain to control GnRH release that drives menstrual cycle, whereas, the activity of the "GnRH surge generator" is primed by the rising follicular phase levels of estradiol to generate the pre-ovulatory surge (Herbison, 2020).

Furthermore, during the follicular development, circulating estrogen fine-tunes pulsatile release of GnRH to keep circulating levels of FSH and LH adequately and to ensure the follicular development, therefore, the circulating levels of estrogen serve as a messenger for transmitting the maturity status of ovarian follicles to the hypothalamus, which plays a pinnacle role in the hierarchical control of the HPG axis in female mammals (Uenoyama *et al*.,2021).

Moreover, the menstrual cycle is the result of a highly coordinated HPG axis with hormonal feedback loops that lead to the formation of a dominant follicle, whereas, FSH is responsible for the recruitment of ovarian follicles and follicular growth, highly coordinated feedback loops result in growth of the development of a dominant follicle during the first phase of the menstrual cycle (Itriyeva,2022).

On the other hand, the present results revealed that FSH levels increased significantly ($p \leq 0.05$) in all days in the second group in comparison with the similar days of the first group (Table 4.1.2, figure 4.1.2.2).

This increase may be explained via the high levels of kisspeptin that enhance GnRH and gonadotropin secretion as the present kisspeptin levels increased significantly in second group in comparison with first group (Table 4.1.2, figure 4.1.1.2), moreover, premenopausal women and those whom inside the menopausal transition characterized with a poor ovarian response to high secretion of gonadotropin that leading to low levels of ovarian steroids as (Table 4.1.2, figure 4.1.4.2, figure 4.1.5.2) mentioned an estradiol and progesterone reduction in these age (second group).

These results are in agreement with many studies. Kisspeptin is a neuropeptide that regulates the secretion of GnRH, which, in turn, acts on the anterior pituitary gland to enhance the secretion of gonadotropin hormones, kisspeptin serum levels increase with age, whereas, its higher in women over 35 years (Ibrahim ,2020).

Furthermore, Barabas and his colleagues 2020 found that during the menstrual cycle, kisspeptin is upstream GnRH which produced by the hypothalamus via GnRH neurons directly, influence the secretion of FSH and LH from the anterior pituitary gland.

Moreover, Kawamura and his colleagues 2020 showed that women 30 to 45 years age have a poor ovarian response and both of high and low levels of FSH and estradiol, respectively.

In addition, Vural and his colleagues 2014 showed that in the poor responder group (< 45 years) FSH and LH levels were higher, but estradiol levels and the number of oocytes were lower in comparison with a younger group.

Furthermore, Luna and his colleagues 2007 showed that low reproductive potential in old women (> 35 years) associated with an elevated FSH levels due to overall poor ovarian responsiveness.

5.3 luteinizing hormone (LH)

The present results revealed that LH levels increased significantly ($p \leq 0.05$) in the 16th day in comparison with the other days in both groups (Table 4.1.1, figure 4.1.3.1).

This day mostly associated with the time of ovulatory period, whereas, more amount of this hormone must be provided, therefore, kisspeptin be higher during the ovulatory period as the present results mentioned (Table 4.1.1, figure 4.1.1.1) , kisspeptin up-regulates GnRH/LH secretion, in addition , this LH elevation may be responsible about the development and maintenance of new corpus luteum simultaneously with prolactin (luteotropic hormones) to secrete more progesterone , notably, the present prolactin and progesterone hormones increased at the 16th of the cycle (Table 4.1.1, figure 4.1.5.1, figure 4.1.6.1).

The present findings and thoughts are in consent with many studies. Johnson and his colleagues 2018 found that, there was a high degree of variability with respect to both menstrual cycle length (23–35 days) and day of ovulation (days 14–19), with the model value being at day 16. Moreover, Eissa and his colleagues 2014 showed that the suitable time of ovulation almost occurs at mid period (10 to 18 days from the beginning of menstrual cycle). In addition, Tatlipinar and his colleagues 2001 found that ovulation takes place on day 14-16 of the menstrual cycle.

Furthermore, Kisspeptin up-regulates GnRH secretion, besides, it is a fundamental component for LH surge and ovulation. (Trevisan *et al*.,2018).

Moreover, Hoskova and his colleagues 2022 showed that the hypothalamic neuropeptide kisspeptin potently stimulates the secretion of GnRH, which in turn stimulating induced LH pulses.

Moreover, LH is a key luteotropic hormone that stimulates ovulation, luteal development, progesterone biosynthesis, and maintenance of the corpus luteum (Przygrodzka *et al*.,2021).

Furthermore, Patricio and Sergio, 2019 showed that granulosa cells become luteinized as a result of an increase in LH, which also enhances the production of progesterone and the development of the corpus luteum.
In addition, the growth and function of corpus luteum are regulated by luteotropic and luteolytic factors, the luteotropic factors mainly include prolactin and LH (Vashistha, 2020).

Moreover, Szawka and his colleagues 2010 showed that kisspeptin can acutely stimulate prolactin secretion, through the suppression of tuberoinfundibular dopamine release, as a consequence, it seems feasible that each episode of kisspeptin release driving an LH pulse may also cause synchronized reduction in dopamine levels at the median eminence, leading to a temporally-linked pulse of prolactin secretion (Grattan and Szawka, 2019).

On the other hand, the present results revealed that LH levels increased not significantly in all days of the second group in comparison with the similar days of the first group (Table 4.1.2, figure 4.1.3.2).

Although this slight elevation, it's worth mentioning that the ending of women reproductive age may be the possible cause of the high levels of gonadotropins, in addition, the synergistic action of the present high levels of kisspeptin and prolactin perhaps beyond of LH elevation (Table 4.1.2, figure 4.1.2.1, figure

4.1.6.2), moreover, the present low levels of estradiol during these age (menopausal transition) (Table 4.1.2, figure 4.1.4.2) may be losses or attenuates the control of LH release via its feedback mechanism ,thereby, un limited amount of LH be secreted.

The present findings and ideas are in agreements with following studies. Talaulikar ,2022 showed that the menopausal transition is characterized by highly variable patterns of gonadotropin and sex steroid output, as women progress through the transition, follicle failure appears to occur and sex steroids production wanes dramatically but intermittently.

Furthermore, Vural and his colleagues 2014 showed that in the poor responder group (< 45 years) FSH and LH levels were higher, but estradiol levels and the number of oocytes were lower in comparison with another younger groups.

Moreover, kisspeptin is expressed in hypothalamic kisspeptin neurons that control GnRH secretion and plays a key role in the central mechanism regulating the HPG axis (Stevenson *et al*.,2022).

Furthermore, Ibrahim and his colleagues 2020 found that kisspeptin serum levels increase with age, whereas, it be higher in women above 35 years than those less 24 years.

In addition, the pulsatile secretion of LH and prolactin occurred at the same time (Masaoka *et al*.,1988), Clarkson and his colleagues 2017 showed that the arcuate KNDy neurons are a common “pulse generator” that drive secretion of the two hormones, whereas, each phasic burst of kisspeptin release from those neurons, stimulating a pulse of GnRH/LH secretion and a subsequent prolactin pulse.

In addition, Weiss and his colleagues 2004 showed that during the menopausal transition, the hypothalamic-pituitary axis appears to lose sensitivity to both positive and negative feedback by estrogen, whereas, levels of estrogen capable of lowering LH in younger women were not able to cause negative feedback of estrogen on LH secretion.

5.4 Estradiol

The present results revealed that estradiol levels increased significantly ($p \leq 0.05$) in the 8^{th} day (except the 16^{th}) in comparison with 24^{th} day of both groups (Table 4.1.1, figure 4.1.4.1).

Estradiol hormones and its relation with HPG axis may be an indicator for the ovarian follicle's maturation, therefore, the follicular phase of the cycle (including 8^{th} day) characterized with these presents high levels of FSH and estradiol hormones (Table 4.1.1, figure 4.1.2.1) that act simultaneously on the ovarian follicle's growth and maturation.

The present results are in agreement with many thoughts and studies. During follicular development circulating estrogen fine-tunes the pulsatile release maintain adequate circulating levels of GnRH and gonadotropins, whereas, under FSH stimulation, ovarian follicles grow to a large and mature size, estrogen production and release gradually increase along with follicular development, therefore, the circulating levels of estrogen act as a messenger to transmitting the maturity status of ovarian follicles to the hypothalamus, which controls the HPG axis in a hierarchical fashion (Uenoyama *et al*.,2021).

Moreover, many researchers pointed that an elevation for estradiol hormone during the first few days of the follicular phase resulted from granulosa cells of

the follicle stimulating by circulating levels of FSH rise to secrete estradiol, whereas, estradiol levels continuing to rise as follicles mature (Patricio and Sergio,2019; Itriyeva, 2022).

In addition, estradiol increases slowly in a progressive manner thanks to the increased FSH progress, to the recruitment and growth of follicles (Weishaar,2022).

On the other hand, the present results revealed that estradiol levels decreased not significantly in all days of the second group in comparison with the similar days of the first group (Table 4.1.2, figure 4.1.4.2).

However, this slight present reduction in ovarian estradiol, the premenopausal and menopausal transition women (second group) have a poor ovarian response with the present high levels of gonadotropins (FSH, LH) (Table 4.1.2, figure 4.1.2.2, figure 4.1.3.2) stimulation.

Kawamura and his colleagues 2020 showed that women aged 30 to 45 years associated with a poor ovarian response had elevated serum FSH levels as well as low serum estradiol levels.

In addition, Vural and his colleagues 2014 showed that FSH and LH levels were higher in the poor responder group (< 45 years), but estradiol levels and oocyte count were lower in comparison with other younger groups.

Furthermore, in premenopausal women, concentrations of estrogens are lower than seen in earlier years which correlates with the depletion of follicles in the ovaries and the ovarian resistance to gonadotropins associated with a reduction in estrogen production (Honour,2018).

Moreover, Randolph and his colleagues 2011 found that serum FSH and estradiol changes across the menopausal transition were associated with age, whereas, premenopausal women appeared to have a higher serum FSH and slightly lower serum estradiol levels.

Furthermore, Moreau and his colleagues 2020 found that, estradiol hormone declines progressively across stages of the menopause transition until reaching menopause.

5.5 Progesterone

The present results revealed that progesterone levels increased significantly ($p \leq 0.05$) in the 24th day in comparison with other days in both groups (Table 4.1.1 ، figure 4.1.5.1).

The luteal phase of the cycle (including the 24th day) is characterized by the presence of a corpus luteum that secretes the current high levels of progesterone and some estradiol, furthermore, the deficiency in the present kisspeptin and gonadotropins levels (during the 24th day) (Table 4.1.1, figure 4.1.1.1, figure 4.1.2.1, figure 4.1.3.1) may be reflect the current high level of progesterone.

These results and ideas are in agreements with following studies. Itriyeva ,2022 showed that luteal phase is usually 14 days long in most women, after ovulation, the corpus luteum is forming, which it's a transient endocrine organ that predominantly secretes progesterone. Moreover, Mesen and Young,2015 showed that the luteal phase occurs after ovulation and corresponds to the time when a functioning corpus luteum secretes progesterone.

Furthermore, "GnRH pulse generator" is restrained by post-ovulation progesterone secretion to bring luteal phase slowing of pulsatile gonadotropin release (Sleiter *et al*.,2009; Herbison, 2020).

Moreover, Szeliga and his colleagues 2022 showed that progesterone is the main contributing factor responsible for a reduction in LH pulse frequency in the luteal phase. Furthermore, KNDy neurons mediate the negative feedback actions of both progesterone and estradiol, with dynorphin and kisspeptin, respectively, being the primary outputs producing these effects,whereas, progesterone inhibits GnRH pulse frequency via increased dynorphin release from KNDy neurons which acts within the KNDy network and directly on GnRH neurons (Nestor *et al*.,2018).

On the other hand, the present results appeared a decrease tendency in progesterone level at the all days of the cycle of the second group in comparison with the similar days of the first group (Table 4.1.2, figure 4.1.5.2).

However, this slight present reduction in ovarian progesterone, the premenopausal and menopausal transition women (second group) have a declining in both number of follicles and ovarian response that associated with high levels of kisspeptin (Table 4.1.2, figure 4.1.1.2), gonadotropins (FSH, LH) (Table 4.1.2, figure 4.1.2.2, figure 4.1.3.2) and low levels of estradiol (Table 4.1.2, figure 4.1.4.2).

These results and ideas are in agreements with following studies. Serum progesterone tended to be lower in older animals (Mara *et al*.,2020), this is consistent with what is observed in a human population, as progesterone tends to decline with reproductive age and demonstrates a relationship between this decline and ovarian reserves (Pal *et al*.,2010).

Furthermore, the number of ovarian antral follicles has been reported to be decreased significantly in women 22–42 years aged leading to some reproductive deviations that cased by the aging of reproductive organs, thereby a follicular phase shortening with high estrogen level and low progesterone level during follicular and luteal phases, respectively (Santoro *et al*.,2003).

Moreover, progressive loss of ovarian follicles accompanied by a decrease in progesterone level during aging, this aging characterized by wackiness of estradiol feedback mechanism on both hypothalamus and pituitary (Hall,2007).

In addition, aged mice have a low number of growing follicles and corpora lutea that means a different changes in ovarian sex steroids (low levels of estradiol and progesterone) and high levels of gonadotropins (high levels of FSH and LH) (Wang *et al.*, 2018).

5.6 Prolactin

The present results revealed that prolactin levels increased significantly ($p \leq 0.05$) (except 24th day) in the 16th day in comparison with 8th day in both groups (Table 4.1.1, figure 4.1.6.1).

The 16th day of the cycle may be demonstrated the time of the ovulatory period and around it, the elevation of prolactin during this time associated with more both present kisspeptin (as a LH promoter and as a dopamine antagonist) and LH (as an ovulatory agent) (Table 4.1.1, figure 4.1.1.1, figure 4.1.3.1), beside that, both of prolactin and LH have the same origin release and they considered as a luteotropic hormones, thereby, they act at the same route.

The present findings and thoughts are in consent with many studies. Johnson and his colleagues 2018 found that, there was a high degree of variability with

respect to both menstrual cycle length (23–35 day) and the time of ovulation (14–19 day). Moreover, Eissa and his colleagues 2014 showed that the ovulation times almost in the middle of the period (between 10 - 18 days from the beginning of menstrual cycle).

Furthermore, Aquino and his colleagues 2019 showed that kisspeptin beside its pivotal role in LH control, it's also influencing prolactin secretion, its regulation of prolactin secretion may involve at the concurrent reduction in dopamine activity (the main hypothalamic regulator prolactin substance secretion), which probably facilitates prolactin secretory response.

In addition, the pulsatile secretion of LH was associated with concurrent pulses of prolactin (Masaoka *et al*.,1988), suggesting a common "pulse generator" was driving secretion of the two hormones, the arcuate KNDy neurons could certainly act as pulse generator for both hormones, such that each phasic burst of kisspeptin release from those neurons, triggering a pulse of GnRH/LH secretion and a subsequent prolactin pulse (Clarkson *et al*.,2017) , in addition, kisspeptin may simultaneously reduce dopamine output from the median eminence and induce a pulse of prolactin secretion (Grattan and Szawka,2019).

In addition, the growth and function of corpus luteum are regulated by luteotropic and luteolytic factors, the luteotropic factors mainly include prolactin and LH (Vashistha, 2020).

On the other hand, the present results revealed that prolactin levels increased not significantly in all days of the second group in comparison with the similar days of the first group (Table 4.1.2, figure 4.1.6.2).

This slight elevation can be explained by its correlation with the current high levels of kisspeptin (dopamine's inhibition) during menopause transition(second group) (Table 4.1.2, figure 4.1.1.2), furthermore, the current prolactin may be associated with the present decline in estradiol levels (Table 4.1.2, figure 4.1.4.2) (via disturbing the ovarian function) and with the current decline in progesterone levels (Table 4.1.2, figure 4.1.5.2) (via the early corpus luteum premature regression). It is worth mentioning that menopause transition characterized with the hormonal heterogeneity, thereby, it is not easy to clarified an integrated hormonal picture in these premenopausal women.

These results and findings are in agreement with the observations of many studies. Serum prolactin levels are increase concurrently with elevated serum kisspeptin levels in aged women (Ibrahim,2020), whereas, kisspeptin increases prolactin secretion through inhibition of TIDA neuron (Sawai *et al*.,2014). Moreover, kisspeptin increases prolactin secretion through inhibition of TIDA neurons (Ribeiro *et al*.,2015).

In addition, prolactin levels increased with advancing age in rats, the contacts between TIDA neurons and kisspeptin neurons maintained after reproductive aging (Iwata *et al*.,2016).

Furthermore, Auriemma and his colleagues 2020 showed that prolactin levels have unfavorable effects on ovarian functions via their direct inhibitory effect on the ovaries, leading to decreased synthesis of estrogen and progesterone.

Moreover, Pałubska and his colleagues 2017 showed that the increase in serum prolactin concentration causes disturbance in the function of the gonads by weakening the development of the follicles and decreasing the estradiol production from the granulosa cells, in addition, high levels of prolactin are

causing the premature regression of the corpus luteum and decreased the progesterone levels.

In addition, Landgren and his colleagues 2004 showed that the hormonal characteristics of menstrual cycles in healthy women approaching menopause (menopause transition) are heterogeneous.

5.7 Hydrogen peroxide (H_2O_2)

The present results revealed that H_2O_2 levels trend to increase in the 16^{th} day in both groups (Table 4.2.1, figure 4.2.1.1).

Ovulation is an inflammatory process that characterized by high levels of ROS (such as present H_2O_2) , high levels of cytokines (such as present G-CSF. Table 4.2.1, figure 4.2.4.1) and the present high levels of WBC (Table 4.3.1, figure 4.3.1.1) , thereby , H_2O_2 be increased during ovulation period , in addition , H_2O_2 mimic the effects of LH via series of steps to ensure successful ovulation and corpus luteum development , furthermore, this increase associated with the present high levels of kisspeptin , LH , prolactin hormones (Table 4.1.1, figure 4.1.1.1, figure 4.1.3.1 and figure 4.1.6.1) and HDL (Table 4.4.1, figure 4.4.3.1) that contribute together to keep a successful ovulation. In addition, kisspeptin decreases ROS generation, oxidative stress and increases levels of antioxidants by activates several steps.

The results are in agreement with many studies. Ovulation has several similarities with inflammation and is closely connected to the activity of leukocytes and inflammatory cytokines, moreover, ROS are massively generated during the inflammatory process and could be involved in the signaling cascade, leading to ovulation, in addition, the effect of LH in follicle rupture, mimicked by H_2O_2, whereas, LH activates multiple signaling pathways, including protein kinase A (PKA), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K)

and p38MAPK, ending by release of the oocyte (Yanagi *et al*.,2002; Fujii *et al*.,2005; Shkolnik *et al.*, 2011; Ciani *et al*.,2015; Duffy *et al*.,2019).

ROS are generated by the corpus luteum, which plays a major role in progesterone synthesis, when ROS are produced excessively, oxidative damage to the corpus luteum impairs the generation of progesterone (Al-Gubory *et al*.,2012; Wang *et al*.,2017).

It's worth noting that kisspeptin , LH , prolactin hormones and HDL work together to regulate the ovulation process , whereas , kisspeptin is GnRH upstream directly influence the secretion of LH from the anterior pituitary gland , therefore , the LH surge that occurs at the mid-cycle is responsible for the start of ovulation , moreover , each phasic burst of kisspeptin release from KNDY neurons, triggering a pulse of GnRH secretion and a subsequent LH pulse (Clarkson *et al*.,2017), in addition , kisspeptin may simultaneously reduce dopamine output from the median eminence and induce a pulse of prolactin secretion (Grattan and Szawka,2019), in addition , Hamdi and his colleagues 2010 mentioned that HDL are the main lipoproteins present in human follicular fluid and are important in fertilization and human reproduction.

Furthermore, kisspeptin overexpression alleviated the accumulation of ROS and enhanced the levels of the antioxidants in ovarian granulosa cells, through activation of the PI3K/AKT signaling pathway (Huang *et al*.,2021; Sun *et al*.,2023).

On the other hand, the present results revealed that H_2O_2 in the second group increased significantly ($p \leq 0.05$) in comparison with the first group (Table 4.2.2, figure 4.2.2).

This increase may be attributed to the ovarian aging related with advancing age, that considered as low- grade inflammation characterized by high levels of

pro- inflammatory cytokines (present G-CSF, table 4.2.2, figure 4.2.4.2), ROS (present H_2O_2) and the present WBC (Table 4.3.2, figure 4.3.2). In addition, the present low estradiol (second group, table 4.1.2, figure 4.1.4.2) may be caused H_2O_2 increase due to its low anti-oxidantal effect.

These results and thoughts are in agreement with many studies. The balance (ROS and antioxidants) is broken toward an overabundance of ROS during advancing age, thereby, oocyte aging and gradual decrease of oocyte quantity and quality, therefore, oxidative stress may act as the "initiator" for oocyte aging (Miyamoto *et al*., 2010; Wang *et al*., 2022).

Moreover, advancing age considered as low- grade inflammation and characterized by high levels of pro- inflammatory cytokine and ROS production (such as H_2O_2) (Korolchuk *et al*., 2017; Gorgoulis *et al.*,2019; Saavedra *et al.*,2023).

Advancing age characterized by reduced reproductive function, accompanied by decreased levels of estrogen, which an established antioxidant in the body, thus leads to oxidative stress in various tissues due to the release of ROS (Randolph *et al*.,2011; Doshi and Agarwal, 2013; Chon *et al*.,2021).

5.8 Catalase (CAT)

The present results appeared a high CAT levels tendency in the 8^{th} day of the cycle for both groups (Table 4.2.1, figure 4.2.2.1).

This increase during follicular phase may be attributed to maintenance and protection of the developing follicles against oxidative stress attack resulting by increase ROS levels that need high amount of CAT to neutralize them, CAT

increase accompanied with presence of FSH and estradiol (Table 4.1.1, figure 4.1.2.1, figure 4.1.4.1) which acts as a folliculogenesis agent and as anti-oxidant factor.

These results and ideas are in agreements with following studies. CAT activity was augmented during follicle growth and there was a lack of an increase in ROS levels in follicular fluid during follicle development, which ensures oocyte protection from oxidative stress (Basini *et al*.,2008; Gupta *et al.*,2011; Paine *et al*.,2013; Wang *et al*.,2017; Akhigbe *et al*.,2022).

Changes in the antioxidant enzyme CAT and estradiol activity of ovarian follicular cells in various stages of development fluctuated with FSH, the concomitant increases in CAT and estradiol in response to FSH refers to their roles in follicular selection, folliculogenesis and prevention of apoptosis, whereas, CAT acts as a protective factor to neutralize H_2O_2 to maintain ROS balance and prevention of apoptosis (Behl and Pandey, 2002; Paine *et al*.,2013; Wang *et al*.,2017).

On the other hand, the present results revealed that CAT decreased significantly ($p \leq 0.05$) in all days of the second group in comparison with the first group (Table 4.2.2, figure 4.2.2.2).

This decrease may be attributed to the ovarian aging related with advancing age (considered as low- grade inflammation characterized by low levels of antioxidants (CAT) and high oxidative stress) and to the low levels of estradiol in the second group (Table 4.1.2, figure 4.1.4.2).

The present findings and ideas are in agreements with following studies. The reproductive aging accompanied by a change in the antioxidant enzymatic pattern (such as CAT) in follicular fluid that impaired ROS scavenging efficiency,

therefore, CAT have a negative relation with the progressive age (Ahmed *et al.*,2020; Debbarh *et al.*,2021).

During the process of ovarian aging, there is a gradual increase in follicular ROS levels (H_2O_2), which may result from either an increase in ROS production or a decrease in ROS degradation by antioxidants such as CAT, which are present as the front-line antioxidant defense (Elizur *et al.*, 2014; Ighodaro and Akinloye, 2018).

Advancing age characterized by redaction reproductive function, accompanied with decrease levels of estrogen, that leads to oxidative stress due to the ROS (H_2O_2) release (Randolph *et al*.,2011; Doshi and Agarwal, 2013; Wu *et al*.,2016; Chon *et al*.,2021).

5.9 lecithin- cholesterol acyltransferase (LCAT)

The present results revealed a high LCAT levels tendency in the 8^{th} day of the cycle for both groups (Table 4.2.1 ، figure 4.2.3.1).

The steroidogenesis process (follicular phase) requires sufficient amounts of cholesterol (present TC, table 4.3.1, figure 4.4.1.1) provided by LCAT that considered as a maturation factor for HDL to complete a successful of ovulation, later, in addition, LCAT associated with the present FSH and estradiol levels (Table 4.1.1, figure 4.1.2.1, figure 4.1.4.1) to facilitate folliculogenesis. Furthermore, LCAT may plays a role during this phase as an antioxidant agent to reduces oxidative stress and to ensure an intact oocyte.

The present findings and ideas are in agreements with following studies. During the follicular phase there was an increase in total cholesterol (required for

steroidogenesis), which represent the precursor substrate molecule for all steroid hormones (Oria *et al*.,2020; Bassi *et al*.,2022; Sharma *et al*.,2022).

LCAT plays an important role in reverse cholesterol transport and follicular synthesis of estrogen, high LCAT activity was positively associated with antioxidant accumulation and lower LCAT activity was associated with their consumption, antioxidants are accumulated in the mature follicles to protect LCAT from oxidative damage and promote steroidogenesis (Cigliano *et al*., 2002; Ruder *et al*.,2008; Kunnen and Van, 2012; Cruz *et al*.,2014; Onaolapo *et al*.,2022).

Follicular fluid LCAT activates the apolipoprotein A1 (APOA1) (the major protein of HDL) by gonadotropin-dependent upregulation, thereby, expression of HDL receptors in the ovary cells increase, whereas, HDL is the main lipoproteins present in human follicular fluid and is important in ovulation and fertilization (Balestrieri *et al*.,2001; Adhikari, 2011; Chang *et al*.,2017; Huang *et al*.,2019).

Many studies mentioned that LCAT have a positive relationship with the fertilization rate, catalyzes lipid transporting and decreases oxidative stress that is require for the maintenance and repair of oocyte (Pfrieger and Ungerer, 2011; Buschiazzo *et al*.,2013; Vaughan *et al*.,2014; Wu *et al*., 2016).

On the other hand, the present results revealed that LCAT decreased not significantly in the second group in comparison with the first group (Table 4.2.2, figure 4.2.3.2).

This decrease may be attributed to the ovarian aging related with advancing age (considered as low- grade inflammation characterized by low levels of antioxidants and high oxidative stress) and to the low levels of estradiol (second

group) (Table 4.1.2, figure 4.1.4.2), in addition, its association with the present HDL decrease (Table 4.4.2, figure 4.4.3.2) may be threated the oocyte safety.

These results are in agreement with many studies. LCAT concentrations decrease with advancing age, whereas, advancing age characterized by reduced reproductive function, accompanied by decreased levels of estrogen (an established antioxidant), that leads to oxidative stress due to the ROS release (Randolph *et al*.,2011; Swapnali *et al*.,2011; Doshi and Agarwal, 2013; Chon *et al*.,2021).

Many studies pointed that LCAT decreased with advancing age leading to the important role of LCAT on APOA1 activation, this APOA1 interact with HDL receptor and facilitate to remove cholesterol from the peripheral cells to transport it back to liver (Balestrieri *et al.*,2001; Swapnali *et al*.,2011; Hirashio *et al*., 2014; Wu *et al.*,2016; Dobiasova, 2017; Ko *et al*.,2020).

5.10 Granulocyte colony - stimulating factor (G-CSF) and White blood cells (WBC)

The present results revealed that G-CSF and WBC increased significantly ($p \leq 0.05$) (excepted 8^{th} day) in the 16^{th} day in comparison with 24^{th} day for both groups (Table 4.2.1, figure 4.2.4.1), (Table 4.3.1, figure 4.3.1.1).

Ovulation is an inflammatory process characterized by high levels of pro-inflammatory cytokines (G-CSF), WBC and ROS, in addition, this increase in both G-CSF and WBC associated with high levels of kisspeptin, LH, prolactin hormones (Table 4.1.1, figure 4.1.1.1, figure 4.1.3.1 and figure 4.1.6.1), HDL (Table 4.4.1, figure 4.4.3.1) and H_2O_2 (Table 4.2.1, figure 4.2.1.1) that keep together a guarantee successful ovulation and a good quality oocyte.

The results are in agreement with many studies. Ovulation has several similarities with inflammation and its closely connected to the activity of leukocytes and inflammatory cytokines , whereas , during menstrual cycle , G-CSF gradually boosts during the follicular phase and reaches its peak at ovulation time, G-CSF leads to leukocyte accumulation (in the follicle and follicular wall) and accelerates ovulation , thereby , follicular G-CSF production depends on the paracrine interaction between granulosa cells and leukocytes before ovulation (Hock *et al*.,1997; Kahyaoglu *et al*.,2015; Eftekhar *et al*.,2018 ; Duffy *et al*.,2019 ; Noel *et al*.,2020).

It's worth noting that kisspeptin, LH, prolactin and estradiol hormones involved in successful ovulation process (Clarkson *et al*.,2017; Grattan and Szawka ,2019; Uenoyama *et al*.,2021; Barabas *et al.*,2020). Moreover, during the inflammatory process, ROS are massively produced and may be implicated in the signaling cascade that leads to ovulation, whereas, H2O2 totally duplicates the impact of LH to ensure effective ovulation. (Shkolnik *et al.*, 2011), in addition, Hamdi and his colleagues 2010 mentioned that HDL are the most abundant lipoproteins in human follicular fluid and play a crucial role in fertilization and reproduction.

On the other hand, the present results revealed that G-CSF and WBC increased statistically ($p \leq 0.05$) for the all days of the second group in compared with the first group (Table 4.2.2, figure 4.2.4.2) (Table 4.3.2, figure 4.3.2).

These elevations might be explained according estradiol level deficiency (Table 4.1.2, figure 4.1.4.2) and ovarian aging during premenopause period (second group), that reflect bad quality oocyte and ovulation.

These results and thought are in agreement with the observations of many studies. Ovarian aging is manifests as reproductive decline until the loss of fertility, characterized by the progressive decline in the number and quality of oocytes, accompanied by endocrine dysfunction (Wu *et al*.,2022), in addition, age-related ovarian aging is a natural and inevitable physiological aging process. The age-dependent decline in oocyte quality accelerates between 35 and 40 years, and the natural menopause transition usually occurs between 40 and 45 years (Mishra *et al.*,2019).

Moreover, advancing age considered as low-grade inflammation and characterized by high levels of pro-inflammatory cytokine (such as G-CSF) and ROS production (Korolchuk *et al*., 2017; Gorgoulis *et al.*,2019; Saavedra *et al.*,2023).

Moreover, high levels of G-CSF (major growth factor that drives neutrophil production) to compensation for neutrophil numbers decline caused by senescence of hematopoietic stem cells due to biological aging and estradiol deficiency (Stark *et al*.,2005; Chen *et al*.,2016), this G-CSF increase leads to shorten neutrophil maturation time in the bone marrow and faster release into circulation, thereby, immature neutrophils increase with less functional phenotype (Moss and Rappaport, 2021).

5.11 Lipid Profile

5.11.1 Total cholesterol (TC)

The present results revealed that TC increased non-significantly (except the 24th day) in the 8th day in comparison with the 16th day of the first group (Table 4.4.1, figure 4.3.1.1) and increased non-significantly in the 8th day in comparison with all days of the second group (Table 4.4.1, figure 4.3.1.1).

This elevation, accompanied with the present high levels of FSH (Table 4.1.1, figure 4.1.2.1), may be refer to cholesterol biosynthesis by FSH induction via a complex mechanism starting with FSH and hepatic FSH binding receptors (FSHRs) and ending by cholesterol biosynthesis, in addition, the present increase in estradiol levels (Table 4.1.1, figure 4.1.4.1) may promoted a favorable lipid profile, whereas, estradiol considers as a buffering capacity of cholesterol.

The present findings and ideas are in agreements with following studies. During the follicular phase there was an increase in total cholesterol, correspond to the rise and peak of estrogen (Panigrahi and Panda, 2018; Sharma *et al*.,2022). Guo and his colleagues 2019 showed that FSH regulated cholesterol biosynthesis via a complex mechanism involved FSH / FSHR binding on the hepatocyte surface followed by different steps activation to approche HMGCR (the rate limiting enzyme for cholesterol biosynthesis).

Moreover, Xu and his colleagues 2022 mentioned that FSH activates Gi2 by binding to liver FSHR / β-arrestin-2/Akt pathway, which subsequently inhibits the binding between FoxO1 and SREBP-2, drives HMGCR transcription and de novo cholesterol biosynthesis, resulting in increased cholesterol levels.

Furthermore, during the reproductive years', estradiol promotes a favourable lipid profile, (Chu *et al.*,2003), whereas, Ness and Chambers 2000 showed that estradiol acts to increase hepatic HMG-CoA reductase activity primarily by stabilizing the mRNA. Moreover, Di Croce and his colleagues 1999 showed that estradiol promotes HMG-CoA reductase gene promoter activity and transcription to increase cholesterol biosynthesis.

On the other hand, the current TC results for the all days of second group revealed a non- significant increase in comparison with the similar days of the first group (Table 4.4.2, figure 4.4.1.2).

This elevation may be explained by its correlation with the current high levels of FSH (Table 4.1.2, figure 4.2.2.2) and the current decrease in estradiol levels (Table 4.1.2, figure 4.1.4.2) in the menopausal transition women , whereas, FSH induced cholesterol biosynthesis via a complex mechanism , that ending by de novo cholesterol biosynthesis, in addition, the deficiency in some hormones (including estradiol) led to elevations in serum cholesterol levels , furthermore, the gradual decrease in the ability to remove cholesterol , might be also participate in the age-related disruption of lipid homeostasis, which led to an accumulation of cholesterol and elevated its levels.

The present findings and ideas are in agreements with following studies. In premenopausal women, elevated FSH leads to an unfavorable circulating TC disturbance by regulating hepatic cholesterol biosynthesis in the liver, which could be reversed by blocking FSH to reduces serum cholesterol via inhibiting hepatic cholesterol biosynthesis (Guo *et al.*,2019).

Furthermore, Ness and Chambers 2000 showed that the deficiencies in hormones (including estradiol) that act to increase hepatic HMG-CoA reductase gene expression led to elevations in serum cholesterol levels.

In addition, Trapani and Pallottini 2010 demonstrate that the causes of age-related disruption of lipid homeostasis include the progressively reduction ability to remove cholesterol through conversion to bile acids and the decrease activity of the bile acid rate-limiting enzyme (cholesterol 7α-hydroxylase C7αOH).

5.11.2 Triglyceride (TG)

The present results revealed that TG increased slightly in the 8th day of the cycle for both groups (Table 4.4.1, figure 4.3.1.1).

This day represents the follicular phase of cycle, whereas, the present elevation may be correlated with the present high levels of both FSH and estradiol (Table 4.1.1, figure 4.1.2.1, figure 4.1.4.1) and may be attributed to the role of FSH in promote lipid biosynthesis via a series of steps ending with TG increase, in addition, estradiol may exert its effect on the TG changeable levels due to its role in maintaining TG homeostasis.

These results are in agreement with many studies concerning the role of TG and its relation with the menstrual cycle.

Simeon and his colleagues 2022 found that TG have higher values at the follicular phase due to production of high estrogen levels from developing follicles.

The follicular phase characterized with increase both the follicle stimulating hormone (FSH) and estradiol (Uenoyama *et al*.,2021).

Liu and his colleagues 2015 showed that the effects of FSH are mediated by FSH receptors coupled to the Gai protein, as a result, Ca2+ influx is stimulated, cAMP-response-element-binding protein is phosphorylated and an array of genes involved in lipid biosynthesis is activated.

Moreover, Reusch and Klemm 2002 showed that FSH could promote lipid biosynthesis by the coupling of FSHR to Ca2+-dependent signaling pathway, as a result, cAMP-response-element-binding protein (CREB) a transcription factor

known to elicit pleiotropic effects on lipid biosynthesis, becomes activated and TG accumulates.

In addition, Zhu and his colleagues 2018 showed that FSH treatment increased lipid biosynthesis and lipid droplet formation through the Gai/ Ca2+/cAMP regulatory element-binding protein (CREB) pathway.

Furthermore, Luo and his colleagues 2017 showed that estrogen is an important factor in maintaining TG homeostasis and up-regulates serum concentrations of Apolipoprotein A5 (APOA5), which possessed a strong ability of decreasing serum TG levels.

On the other hand, the present results revealed that a transient increase in TG during the all-days of the second group (Table 4.4.2, figure 4.4.2.2) in comparison with the first group.

However, premenopausal women (second group) accompanied with imbalance of lipid profiles caused by present deficiency of estradiol (Table 4.1.2, figure 4.1.4.2) that plays a prominent role in lipid homeostasis, beside the continual impacts of FSH secretion (Table 4.1.2, figure 4.2.2.2) on the TG increase.

The present findings and thoughts are in consent with many studies. Kawamura and his colleagues 2020 showed that women with 30 - 45 years aged have an increase and decrease in both FSH and estradiol levels, respectively.

Warjukar and his colleagues 2020 found that premenopausal healthy women (with regular menstrual cycles) have significant differences in lipid parameters (TG increase), as a result of estradiol decrease. Moreover, triglyceride have a negative correlation with estrogen in premenopausal women (Swarnalatha and Ebrahim 2012)

Chu and his colleagues 2003 showed that high levels of FSH possibly increased the lipid biosynthesis that leading to TG accumulation, moreover, FSH acts via the previous mechanism (mentioned above) to regulate lipid biosynthesis during progressive age (Liu *et al.*, 2015).

5.11.3 High density lipoprotein (HDL)

The present results revealed that HDL levels increased significantly ($p \leq 0.05$) in the 16th day in comparison with similar days in both groups (Table 4.4.1, figure 4.3.3.1).

This rise (during the ovulatory days and beyond) , is a crucial factor for successful ovulation , presence of HDL is necessary to both approach lipids homeostasis and correct ovulation without any defects during these days , with presence of the present kisspeptin , LH , prolactin and estradiol (Table 4.1.1, figure 4.1.1.1, figure 4.1.3.1, figure 4.1.6.1 and figure 4.1.4.1) that act collectively to regulate the ovulation process , directly (hormonal action) and indirectly (lipid homeostasis). In addition, kisspeptin decreases ROS generation, oxidative stress and increases the antioxidants capacity through several steps activation.

The present findings and thoughts are in line with many studies. Several researches mentioned that HDL levels increased in ovulatory days, during menstrual cycle (Mumford *et al.*,2011; Panigrahi and Panda ,2018; Sharma *et al*.,2022).

HDL generated from excess cellular cholesterol exported by ATP-binding cassette A1 (ABCA1A) which expressed in theca cells and its deficiency inhibits successful ovulation through cholesterol accumulation in the ovarian follicle (Futamata *et al*.,2023). Moreover, Quiroz and his colleagues 2020 found that

HDL and oocyte ABCA1 transporters regulate mouse oocyte cholesterol homeostasis and contribute to female fertility. Furthermore, Hamdi and his colleagues 2010 mentioned that HDL is the main lipoprotein present in human follicular fluid and is important in ovulation and fertilization.

It's worth noting that kisspeptin, LH, prolactin and estradiol hormones involved in successful ovulation process (Clarkson *et al*.,2017; Uenoyama *et al*.,2021; Barabas *et al.*,2020).

Kirby and his colleagues 2010 showed that kisspeptin regulate lipid metabolism in hepatocytes via his classical pathway by binding with GPR54, the phospholipase C can be activated to produce phosphatidylinositol 4,5-bisphosphate (PIP2), inositol triphosphate (IP3) and diacylglycerol (DAG) and to transmit the signal in cell. Moreover, Wang and his colleagues 2018 showed that kisspeptin can regulate lipid metabolism and kisspeptin/KISS1R pathway is a potent enhancer of hepatocytes, whereas, the administration of kisspeptin in rat and cultured hepatocytes leads to an increase in HDL and lipid synthesis.

Chirico and his colleagues 2013 found that prolactin positively associated with the HDL, whereas, prolactin exerts its biological function via cellular

kinases via PI3K/AKT/STAT5 pathway and participates in the regulation of hepatic lipid metabolism (Titchenell *et al*.,2017). In addition, prolactin can exert anti-lipolytic action in human adipocyte by inhibited the mRNA and protein expressions of fatty acid synthase (Brandebourg *et al*.,2007).

Many studies found that estradiol upregulates mRNA expression of the human homolog of the B class, type I scavenger receptor (SR-BI) and the HDL receptor to promotes HDL cholesterol uptake in peripheral tissues (Lopez and McLean 2006; Fukata *et al.*,2014). Moreover, Panigrahi and Panda 2018 mentioned that

estradiol upregulates ABCA1 and APOA1, a most important HDL protein, which enhance HDL production, in addition, estradiol also increasing VLDL synthesis that leading to subsequent decrease in LDL and increase in HDL.

It's worth noting, kisspeptin overexpression alleviated the accumulation of ROS and enhanced the levels of the antioxidants, through activation of the PI3K/AKT signaling pathway (Huang *et al*.,2021; Sun *et al*.,2023).

On the other hand, the present results revealed a low HDL tendency at the all days of the second group in comparison with the similar days of the first group (Table 4.4.2, figure 4.3.3.2).

This deficiency may be attributed to the bad behavior of lipoproteins caused by the low present levels of estradiol (Table 4.1.2, figure 4.1.4.2) (that plays a prominent role in lipids homeostasis) during both transitional and progressive age.

These results and thoughts are in agreement with the observations of many studies. Lipid pattern changed (HDL decrease) with lose of estradiol effect, during the menopausal transition (Swarnalatha and Ebrahim ,2012; Inaraja *et al.*,2020). Wiacek and his colleagues 2011 showed that the differences in the concentration of HDL are a mixture of the menopausal transition and the aging process.

Moreover, HDL level decreased in aging women, due to their a low level of estradiol and a highest activity of hepatic lipase which enhances the uptake and catabolism of HDL (Fatima and Sreekantha, 2017).

However , HDL is not always good cholesterol , large HDL particles were found to become dysfunctional during the menopause transition , whereas, its ability to

promote cholesterol efflux capacity (CEC) from macrophages become weaker (El khoudary *et al*., 2021).

5.11.4 Low density lipoprotein (LDL)

The present results revealed that LDL increased significantly ($p \leq 0.05$) in the 8th day of the cycle for both groups (Table 4.4.1, figure 4.3.4.1).

The behavior of lipoproteins and their relationship with the sex hormones is still unclear, nevertheless, estradiol (perhaps FSH) plays a role in lipid metabolism physiologically due to its benefit impacts, especially, during the growth and repair period (follicular phase), through its ability in lipid homeostasis and maintenance via several routs i.e ….. as a modulator of the hepatic LDL receptors (LDLR), up – regulated the LDL uptake and its ability to prevent PCSK9 mediated LDLR degradation in liver cells.

These results and ideas are in agreement with the following researches. Many studies mentioned that estradiol have physiologically beneficial effects on lipid metabolism during follicular phase (Terzic *et al*.,2009; Mumford *et al*.,2010; Faulds *et al*.,2012; Ko *et al*.,2020).

Several studies indicated that the peak levels of LDL observed during the follicular phase occurred simultaneously with increased levels of estrogen which associated with an improved lipid profile (Mumford *et al*.,2011; VaShiShta *et al*.,2017; Panigrahi and Panda 2018; Sharma *et al*.,2022).

Huang and his colleagues 2019 found that LDL and HDL play important roles in ovarian cholesterol transport, whereas, cholesterol is an important substrate for the synthesis of ovarian sex hormones and follicular development in follicular phase.

Many studies mentioned that regulation of hepatic LDL receptors is carried out by effect of estradiol on lipid metabolism, estradiol works on the hepatocytes via these LDLR and contributes to greater clearing of LDL particles, by this process the serum LDL levels are regulated (VaShiShta *et al*.,2017; Panigrahi and Panda, 2018).

Serum estradiol is a regulator of lipoprotein lipase LPL, that catalyses the hydrolysis of VLDL to IDL and later to LDL , leading to increase LDL levels , estradiol also stimulates the synthesis of LDLR and ultimately regulatory the plasma LDL level (Tanko *et al*.,2005 ; Fatima and Sreekantha,2017 ; Warjukar *et al*.,2020) .

Jia and his colleagues 2022 showed that estradiol controls LDL levels by regulating the plasma proprotein convertase subtilisin/kexin type 9 (PCSK9), which increasing plasma LDL levels by triggering the degradation of LDLR. Moreover, Fu and his colleagues 2020 found that circulating levels of estradiol have a role in controlling LDL levels by its effect on the PCSK9 that binds and controls to LDL receptors by a mechanism that involves activation of the G protein-coupled estrogen receptor (GPER) which prevents PCSK9-dependent LDLR degradation, leading to increase LDL levels.

Zhang and his colleagues 2020 found that serum FSH positively correlated with LDL levels. Furthermore, Song and his colleagues 2016 mentioned that FSH involved in hepatic LDL metabolism, the increase in FSH levels may attenuated degradation of LDL by inhibited LDLR expression in liver, which in turn could elevate the level of serum LDL. Moreover, Serviente and his colleagues 2019 showed that the LDL elevation might be attributed to FSH increase, whereas, this increase was associated with low LDLR expression.

On the other hand, the present results revealed a high LDL levels tendency in the second group in comparison with the first group (Table 4.4.2, figure 4.3.4.2).

It seemed that the chronological age beside the estradiol deficiency (Table 4.1.2, figure 4.1.4.2) are beyond these lipid (LDL) alterations in these premenopausal women, in addition to the continuing effects of FSH secretion (Table 4.1.2, figure 4.2.2.2) on the LDL increase.

The present findings and thoughts are in line with many studies. Several researchers indicated that in menopausal transition women, deficiency of estradiol, increase activity of plasma LPL causes elevation of LDL along with down regulation of LDLR (Mallick *et al*.,2015; Fatima and Sreekantha,2017; Warjukar *et al*.,2020).

Jia and his colleagues 2022 found that PCSK9 levels change throughout the menstrual cycle and an inverse relation exists between PCSK9 and estradiol in premenopausal women. Therefore, loss of estrogen leading to an increase PCSK9 levels (mediated LDLR degradation), that explain the marked increase of LDL levels in these transition women (Fu *et al.*,2020).

Serviente and his colleagues 2019 showed that FSH elevation associated with an increase in LDL, that increased with decreased LDLR expression. Moreover, Song and his colleagues 2016 mentioned that higher FSH related to higher levels of LDL, whereas, FSH participated in hepatic LDL metabolism via attenuated degradation of LDL and inhibited LDLR expression in liver tissue.

However, Lee and his colleagues 2022 showed that no correlation between FSH and lipid profile. In addition, Jeenduang 2019 mentioned that there was no association between PCSK9 and LDL.

5.11.5 Very low-density lipoprotein (VLDL)

The present results appeared a high VLDL levels tendency in the 16th day of the cycle for the first and the second groups (Table 4.4.1, figure 4.3.5.1).

This tendency accompanied with the current high levels of HDL (Table 4.4.1, figure 4.3.3.1) to provide the ovulation requirements, may be attributed by the present estradiol role (Table 4.1.1, figure 4.1.4.1) in lipid metabolism (VLDL) directly and /or indirectly, as well as, the actions of presence each of kisspeptin, LH and prolactin (Table 4.1.1, figure 4.1.1.1, figure 4.1.3.1 and figure 4.1.6.1) that facilitated ovulation process and may contribute in lipid metabolism.

The present findings and ideas are in agreements with following studies. VLDL levels increased during follicular to ovulatory phase, among menstrual cycle (Gupta *et al.*,2015; VaShiShta *et al*.,2017; Panigrahi and Panda, 2018).

The oocyte (as a rapidly growing cell) has a considerable demand for energy as well as cholesterol, therefore, it appears that ApoB - containing lipoproteins (VLDL) might have a nourishing function that the TG-poor HDL lipoproteins are not able to fulfill properly (Jaspard *et al.*,1997; Stouffer *et al*.,2007; Gautier *et al.*,2010).

Moreover, estradiol exerts a regulatory control for every step of lipid metabolism chain, whereas, estradiol mediates the packaged of circulating fatty acid into TG rich VLDL particles by the liver, in addition, some of estradiol's protective effects in the liver are likely indirectly due to estrogen signaling adipose tissue to limit the release of serum fatty acid that is made into TG is matched with increased VLDL-TG secretion. Moreover, estradiol also increased VLDL levels by regulated LPL, which is responsible for hydrolyzing TG to

chylomicrons and VLDL (Muzzio *et al*.,2007; Chatterjee and Sparks, 2011; Saxena *et al*.,2012; Palmisano *et al.*,2017; Berad, 2019).

Furthermore, kisspeptin, LH and prolactin have a role in lipid metabolism (VLDL), whereas, exogenous KP 10 was associated with the significant elevation in hepatic lipids synthesis and transport in quails, while, LH receptors (LHR) expression is intimately associated with cholesterol transport, synthesis and steroidogenesis in the ovary, whereas, increased prolactin receptors signaling associated with increase in VLDL cholesterol levels (Wang and Menon 2005; Wu *et al*.,2013; van der Sluis *et al.*,2014).

On the other hand, the present results revealed that VLDL increased non-significantly in the second group in comparison with the first group (Table 4.4.2, figure 4.3.5.2).

Although, this slight elevation, the disruption of lipid homeostasis related with progressive age may be caused by the deficiency of estradiol (Table 4.1.2, figure 4.1.4.2) that plays an important role in lipid homeostasis via different mechanisms.

Estradiol lack in menopausal transition women induces relative accumulation of small VLDL particles due to elevate VLDL catabolism resulting in higher number of VLDL residual particles, moreover, the converse implication of estrogen-mediated reductions in fatty acid delivery to the liver and estrogen-mediated increases in VLDL-TG export, leads to fat accumulation (Liu *et al*.,2015; Fatima and Sreekantha, 2017; Palmisano *et al*., 2017; Berad, 2019; Warjukar *et al.*,2020).

Chapter Six
Conclusions and Recommendations

6.1 Conclusions

The results of the present study included the following conclusions:

1. The menstrual cycle and its phases considered as a physiological independent event that act collectively and or separately to influenced the whole-body parameters.
2. According to its actions kisspeptin regulate hypothalamic pituitary gonads axis, it mediates and controls a network of hormones, oxidants / antioxidant, pro inflammatory marker and lipides that mange and regulate the ovulation process and all its related aspects during the menstrual cycle.
3. Successful ovulation reflects the coordination state among different physiological, immunological and oxidantal factors that improves different challenges for the ovulating women.
4. . Estradiol considered as a magic hormone that plays via its different actions separately or synchronizingly with other previous parameters in keep and maintenance the ovarian function thereby enhances the physiological and immunological events that persist along the woman reproductive life.
5. The deterioration hormonal and biochemical studied parameters in old women and their detrimental effects caused by the low-grade Inflammation may be reflected the reproductive performance reduction in these women in contrast with the high reproductive performance in young women.
6. Ages between 40 - 45 years represent a transition period that pointed out a deterioration in all studied parameters and reflect the damage tissues and organs that drive probably to the ending of reproductive age.
7. The studying and evaluating the lipid profile changes according to its phase of menstrual cycle represents an important issue to knowing the physiological and healthy woman state and to prevent the incidence of cardiovascular diseases. This may help in the management and control of cardiovascular

disorders and other pathological conditions associated with alterations in lipid profile especially the pre-menopausal women.

8. Changes in G-CSF and WBC reflect the inflammatory state during the pre and ovulatory phase of the menstrual cycle to keep a good oocyte quality and enhanced a complete ovulation in young women in contrasted to un favorable oocyte properties with some ovulation disorders in old women.

9. Changes in oxidants and antioxidants in young women reflect favorable effects to complete the requirements of the menstrual cycle and its phases such as folliclogenesis , steroidogenesis , ovulation and corpus luteum formation and maintenance in comparison with a detrimental effects caused by inflammaging, ovarian aging and high oxidative stress with low antioxidants in old women .

10. The premenopause (that leading to menopause) is accompanied with potential a diverse changes in hormonal, oxidantal, inflammatoril and lipid profile states independent of any direct effects of aging.

6.2 Recommendations

Further studies should be recommended as following:

1.Extensive studies concerning the menstrual cycles including more samples, vital parameters and periods during different ages.

2.Studying the correlation between kisspeptin and its impacts on other aspects of reproduction and fertility such as fertilization, implantation, pregnancy, and infertility …. etc .

3.Investigate the kisspeptin roles in some metabolic syndromes, i.e …. insulins resistance, obesity, diabetes mellites and hypertension.

4.More studies that aimed to management and control the pathological disorders and conditions associated with fluctuation in lipid profile during pre and postmenopuse .

5.To investigate the roles of neurokinin and dynorphin on the human reproductive field specially in the menstruating women due to their similar kisspeptin 's synthesis and release site.

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استمارة استبيان خاصه ببحث الطالبه نورا كريم عبد الحسن

- رقم العينه .

- محل وتاريخ الولاده.

- الحاله الاجتماعيه ☐ متزوجه ، ☐ غير متزوجه

في حالة الاجابه (متزوجه) هل لديك أطفال .

- عنوان السكن.

- رقم الهاتف .

- هل سبق وان اصبت بأحد الامراض المزمنه مثل ارتفاع ضغط الدم، السكري .

- هل سبق وان اجريت عمليه جراحيه.

- هل تستخدمين موانع الحمل الهرمونيه او غير الهرمونيه .

- هل دورتك الشهريه منتظمه ☐ نعم ، كلا ☐

في حالة الاجابه بكلا ، ما هي علامات عدم الانتظام .

- هل سبق وان اصبت بمتلازمة تكيس المبايض او قصور الغده الدرقيه

- هل انت من المدخنين.

- هل توافقين على عملية سحب الدم لثلاث مرات خلال مراحل الدوره الشهريه .

- هل توافقين على الانضمام الى عينة البحث ☐ نعم ، ☐ كلا

علماً بأن لك الحق في الانسحاب من اكمال هذه المهمه وقت ما تشائين.

الاسم والتوقيع والتاريخ

المستخلص

هدفت الدراسة الحالية الى تقدير مستويات هرمون الكسبتين(Kisspeptin) وعلاقتها ببعض المعايير الهرمونية والبايوكيميائية الاخرى خلال مراحل الدورة الشهرية للنساء في محافظة ميسان. اشتملت عينة الدراسة على ثلاثين امرأة سليمة (أعمارهن ٢٠ - ٤٥ سنة) من اللواتي يراجعن مستشفى الولادة والطفل التخصصي وبعض المراكز الصحية، قسمت العينة الى مجموعتين (١٥ امرأ ة / مجموعة) حسب أعمارهن وعلى النحو الاتي:

- المجموعة الأولى: ٢٠ -٢٥ سنة.
- المجموعة الثانية: ٤٠- ٤٥ سنة.

تم سحب الدم في الأيام الثامن، السادس عشر والرابع والعشرين من الدورة الشهرية.

أظهرت النتائج ما يأتي:

١-ارتفاع مستوى الكسبتين(Kisspeptin) معنوياً (أ≤٠,٠٥) في اليوم السادس عشر مقارنة مع الرابع والعشرين وغير معنوياً مع اليوم الثامن للمجموعة الأولى والثانية على التوالي. كما ارتفع الكسبتين معنوياً (أ≤٠,٠٥) في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

٢- ارتفاع مستوى الهرمون المحفز للحويصلات (FSH) معنوياً (أ≤٠,٠٥) في اليوم الثامن (عدا اليوم السادس عشر) للمجموعة الأولى مقارنة مع اليوم السادس عشر للمجموعة الثانية واليوم الرابع والعشرين لكلا المجموعتين. كما ارتفع معنوياً (أ≤٠,٠٥) في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

٣- ارتفاع مستوى الهرمون اللوتيني (LH) معنوياً (أ≤٠,٠٥) في اليوم السادس عشر مقارنة مع اليوم الثامن واليوم الرابع والعشرين لكلا المجموعتين. كما ارتفع وبشكل غير معنوي في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

٤-ارتفاع مستوى الاستراديول (Estradiol) وبشكل غير معنوي في اليوم الثامن مقارنة مع اليوم السادس عشر، ومعنوياً (أ≤٠,٠٥) مقارنة مع اليوم الرابع والعشرين لكلا المجموعتين. كما انخفض وبشكل غير معنوي في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

٥-ارتفاع مستوى البروجستيرون(Progesterone) معنوياً (أ≤٠,٠٥) في اليوم الرابع والعشرين مقارنة مع اليوم الثامن واليوم السادس عشر لكلا المجموعتين. كما انخفض وبشكل غير معنوي في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

المستخلص

٦-ارتفاع مستوى البرولاكتين(Prolactin) معنوياً (أ≤٠,٠٥) في اليوم السادس عشر مقارنة مع اليوم الثامن، وغير معنوياً مقارنة مع اليوم الرابع والعشرين لكلا المجموعتين. كما ارتفع وبشكل غير معنوي في المجموعة الثانية مقارنة بالمجموعة الأولى وللأيام المتماثلة.

٧-ارتفاع مستوى بيروكسيد الهيدروجين(H_2O_2) وبشكل غير معنوي في اليوم السادس عشر مقارنة مع اليوم الثامن ،ومعنوياً (أ≤٠,٠٥) مع اليوم الرابع والعشرين لكلا المجموعتين. كما ارتفع معنويا(أ≤٠,٠٥) ًفي المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

٨- ارتفاع مستوى انزيم الكتالايز(CAT) وبشكل غير معنوي في اليوم الثامن مقارنة مع اليوم السادس عشر، ومعنوياً (أ≤٠,٠٥) مع اليوم الرابع والعشرين لكلا المجموعتين. كما انخفض معنوياً (أ≤٠,٠٥) في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

٩- ارتفاع مستوى انزيم الليسيثين كوليسترول اسل ترانسفيريز(LCAT) وبشكل غير معنوي في اليوم الثامن مقارنة مع اليوم السادس عشر، ومعنوياً (أ≤٠,٠٥) مع اليوم الرابع والعشرين لكلا المجموعتين . كما انخفض بشكل غير معنوي في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

١٠- ارتفاع مستوى العامل المنبه لمستعمرات الخلايا الحبيبية (G-CSF) وبشكل غير معنوي في اليوم السادس عشر مقارنة مع اليوم الثامن ،ومعنوياً(أ≤٠,٠٥) مع اليوم الرابع والعشرين لكلا المجموعتين. كما ارتفع معنوياً(أ≤٠,٠٥) في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

١١- ارتفاع عدد كريات الدم البيضاء (WBC) وبشكل غير معنوي في اليوم السادس عشر مقارنة مع اليوم الثامن، ومعنوياً (أ≤٠,٠٥) مقارنة مع اليو م الرابع والعشرين لكلا المجموعتين. كما ارتفع معنوياً (أ≤٠,٠٥) في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

١٢- ارتفاع مستوى الكوليسترول الكلي (TC) وبشكل غير معنوي في اليوم الثامن مقارنة مع اليوم السادس عشر، ومعنوياً(أ≤٠,٠٥) مقارنة مع اليوم الرابع والعشرين لكلا المجموعتين. كما ارتفع وبشكل غير معنوي في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

١٣ - ارتفاع مستوى ثلاثي الغليسيريد (TG) وبشكل غير معنوي في اليوم الثامن مقارنة مع اليوم السادس عشر، ومعنوياً (أ≤٠,٠٥) مقارنة مع اليوم الرابع والعشرين لكلا المجموعتين. كما ارتفع وبشكل غير معنوي في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

١٤-ارتفاع مستوى البروتين الدهني عالي الكثافة (HDL) معنوياً (أ≤٠,٠٥) في اليوم السادس عشر مقارنة مع اليوم الثامن واليوم الرابع والعشرين لكلا المجموعتين. كما انخفض معنوياً (أ≤٠,٠٥) في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

١٥- ارتفاع مستوى البروتين الدهني منخفض الكثافة (LDL) معنوياً (أ≤٠,٠٥) في اليوم السادس عشر مقارنة مع اليوم الثامن واليوم الرابع والعشرين لكلا المجوعتين. كما ارتفع وبشكل غير معنوي في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

المستخلص

١٦-ارتفاع مستوى البروتين الدهني المنخفض الكثافة جدا (VLDL) وبشكل غير معنوي في اليوم السادس عشر مقارنة مع اليوم الثامن، ومعنويا (أ≤٠,٠٥) مقارنة مع اليوم الرابع والعشرين لكلا المجموعتين. كما وارتفع وبشكل غير معنوي في المجموعة الثانية مقارنة مع المجموعة الأولى وللأيام المتماثلة.

تمت مناقشة الابعاد الفسيولوجية لهذه النتائج وفقاً لتأثيرات مراحل الدورة الشهرية والتقدم بالعمر الذي يفضي الى انخفاض كفاءة المبيض وما يتبع ذلك من تغيرات هرمونية، تأكسدية وبايوكيميائية.

وخلصت الرساله إلى أن الكسبتين يتوسط ويتحكم في شبكة من الهرمونات ،المؤكسدات / مضادات الأكسدة، علامات الالتهاب وملف الدهون التي تدير وتنظم عملية التبويض وكافة الجوانب المتعلقه بها خلال الدوره الشهريه.

المستخلص

وزارة التعليم العالي والبحث العلمي

جامعة ميسان

كلية العلوم

قسم علوم الحياة

تقدير الكسببتين وعلاقته ببعض العوامل الهرمونية والكيميائية الحيوية خلال المراحل المختلفه للدوره الشهريه في نساء محافظة ميسان

رسالة مقدمة

الى مجلس كلية العلوم/ جامعة ميسان

وهي جزء من متطلبات نيل درجة الماجستير علوم في علوم الحياة

من قبل

نورا كريم عبد الحسن

بكالوريوس تربية/ علوم حياة (2006)

بأشراف

أ. د. احمد عبود خليفة

اب2023م صفر 1445 هـ