

**Republic of Iraq
Ministry of Higher Education
and Scientific Research
University of Thi-Qar
College of Science**



Clinical and Genetic Study for Oxidation-Antioxidation in Women with Unexplained Infertility and Polycystic Ovary Syndrome

Submitted to the Council of the College of Science, University of Thi-Qar in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Clinical Biochemistry

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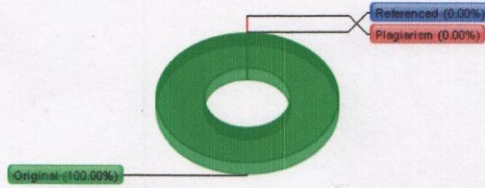
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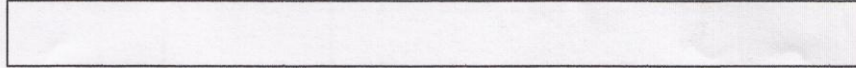
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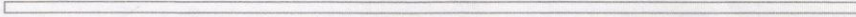


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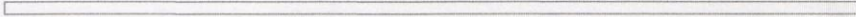
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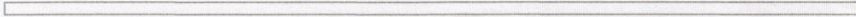
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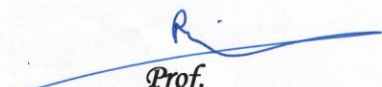
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CERTIFICATE

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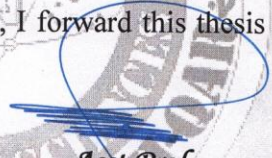

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Dedication

This dissertation is dedicated to God Almighty who has been my eternal rock and source of refuge.

I dedicated this humble effort, the yield of my thoughts and study to the soul of my lovely sister “Nada'a”, whose inspired me to higher ideas of my life. I miss her every day, but I am glad to make her dream come true.

I dedicated this dissertation to the soul of my affectionate Father who the first teaches me.

I also take this opportunity to dedicate this dissertation to my great affectionate mother whose prayers and love took me to the zenith of glory and transformed my dreams into reality.

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List of Abbreviations

Abbreviation	Mean
AFLP	Amplified fragment length polymorphism
AOD	Antioxidant defense system
ATP	Adenosine triphosphate
ASRM	American Society for Reproductive Medicine
AUA	American Urological Association
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
BMI	Body Mass Index
BOH	bad obstetric history
Cp	Ceruloplasmin
CTAB	cetyltrimethylammonium bromide
CVD	Cardiovascular diseases
ddH ₂ O	Deionized distil water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DTNB	5,5-Dithio bis(2-nitrobenzioc acid)
EMC	Erythrocyte membrane cholesterol
eNOS	Endothelial nitric oxide synthase
FSH	Follicle stimulating hormone
GnRH	Gonadotropin Releasing Hormone
GPx	Glutathione peroxidase
G250	Green 250
GSH	Glutathione
GSR	glutathione disulfide reductase
GSSG	Oxidized glutathione
H ₂ O ₂	Hydrogen peroxide
HDL	High density lipoprotein
HG	Hyperandrogenemia
HMG- COA	Hydroxy-3-methylglutaryl co-A
HPO	Hypothalamo-Pituitary-Ovarian
HPT	The hypothalamus-pituitary-thyroid
HWE	Hardy-Weinberg equilibrium
IR	Insulin resistance
IWB	Instruction window buffer
LDL	Low density lipoprotein
LH	Luteinizing hormone
LPO	Lipid peroxidation
MDA	Malondialdehyde

mRNA	Massinger ribonucleic acid
Na ₂ HPO ₄	Disodium hydrogen phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH ₂ PO ₄	Sodium dihydrogen phosphate
NBT	Nitro Blue Tetrazolium
NCBI	National Center Biotechnology Information
NO	Nitric oxide
O ₂ ⁻	Superoxide
POF	Peritubal ovarian failure
PAP	poly (ADP-ribose)
PBS	Phosphate buffer solution
PCOS	polycystic ovarian syndrome
PCR	Polymerase chain reaction
PPD	Para-Phenylenediamin
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNase	Ribonuclease
PRL	Prolactin
RNS	Reactive nitrogen species
ROOH	Organic hydroperoxid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SSA	sub Saharan Africa
StAR	Steroidogenic acute regulatory protein
T3	Triiodothyronine
T4	Tetraiodothyronine
TAC	Total antioxidant capacity
Taq	Thermus aquaticus
TBA	Thiobarbutaricacid
TCA	Trichloroaceticacid
Tf	Transferrin
TG	Tiglycerides
TSH	Thyroid stimulating hormone
U	Unit
UI	Unexplained infertility
UV	Ultra-Violate
VLDL	Very low density lipoproteins
WHO	World Health Organization
XOD	Xanthine oxidase

Summary

This study was designed to investigate the pathogenic role of fertility hormones, oxidative stress, antioxidants, lipid profile levels, some trace elements and glutathione disulfide reductase (GSR) polymorphism in women with unexplained infertility and polycystic ovary syndrome in Iraq (Thi-Qar government) . Sixty eight women with unexplained infertility (UI) as well as (Sixty eight) women with polycystic ovarian syndrome (PCOS) aged (20-35) year were taken from the infertility Unit in Hussrein Teaching Hospital in Nassyrieh. The women were divided according to the type of infertility into primary unexplained (1°UI), unexplained (2°UI), primary polycystic ovarian syndrome (1°PCO) and secondary polycystic ovarian syndrome (2°PCO) groups. Thirty four healthy fertile women with the same age were included in this study as a control group.

The results showed a significant increase ($p \leq 0.05$) in serum prolactin (PRL), thyroid stimulating hormone (TSH) and luteinizing hormone (LH) in groups of women with (PCOS) infertility compared to the control and women with unexplained infertility (UI) groups, and a significant increase ($p \leq 0.05$) in serum malondialdehyde (MDA) levels in all infertility groups compared to control group. Whereas serum levels of the antioxidants glutathione (GSH), superoxide dismutase (SOD) and ceruloplasmin (Cp) were significantly decreased ($p \leq 0.05$) in all infertility groups in comparison with control group. Also the results showed a significant increase ($p \leq 0.05$) in serum total cholesterol (TC), low density lipoproteins (LDL), triglyceride (TG) and very low density lipoproteins (VLDL) and a significant decrease ($p \leq 0.05$) in serum high density lipoproteins (HDL) levels in all infertility groups compared to control group. Also, there were a significant increase ($p \leq 0.05$) in serum (TC,

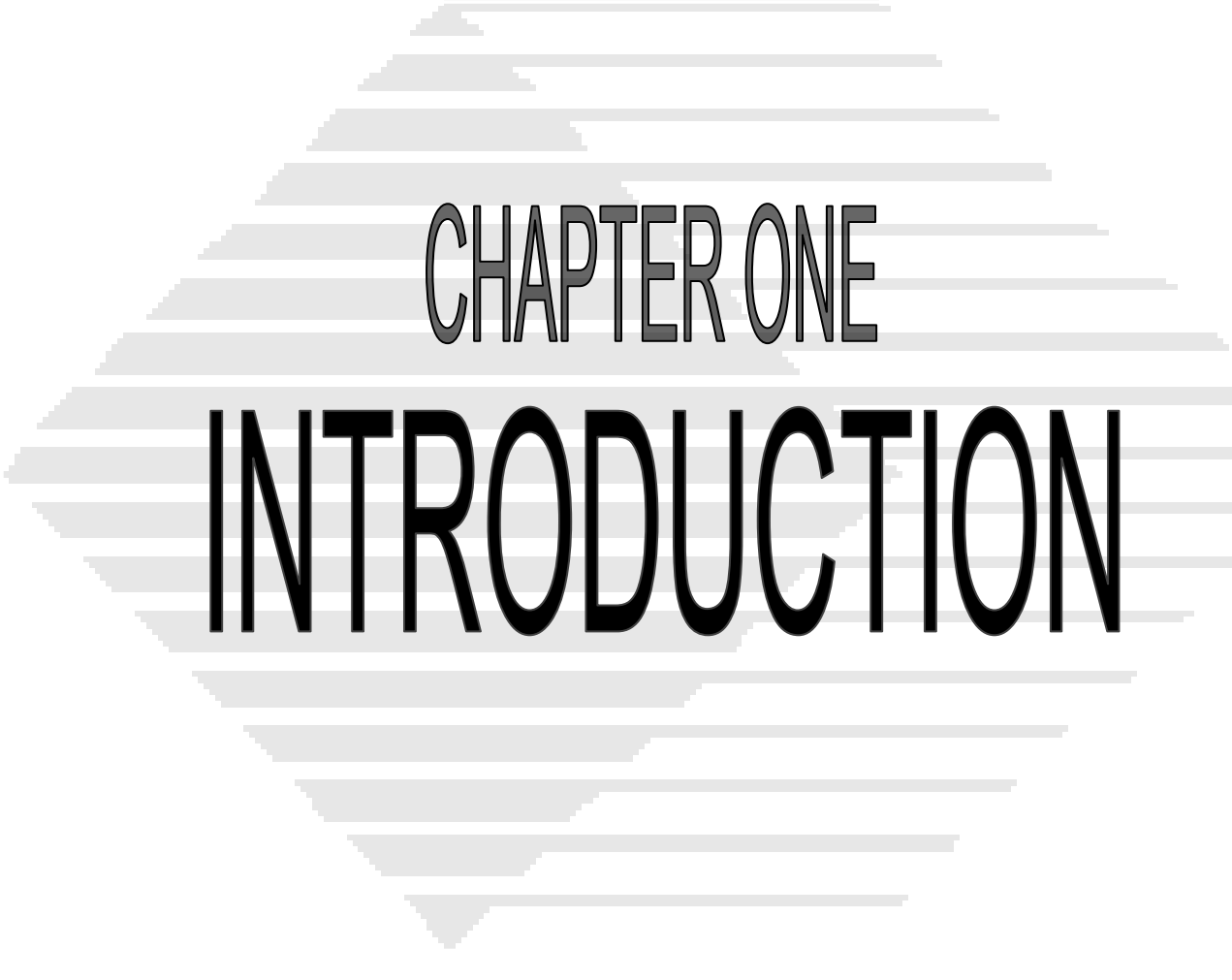
LDL, TG and VLDL) and significant decrease ($p \leq 0.05$) in serum (HDL) in (1°PCO) and (2°PCO) groups in comparison with (1°UI) and (2°UI).

Serum levels of iron and copper significantly increase ($p \leq 0.05$) in unexplained infertility (1°UI) and (2°UI) groups in comparison with PCO infertility and control groups. Whereas no significant differences ($p > 0.05$) were found in iron levels between PCO infertility groups and control. Serum levels of the copper were significantly increased ($p \leq 0.05$) in (PCOS) infertility groups in comparison with control group.

The results obtained from polymerase Chain Reaction (PCR) were done for the detection of (GSR) gene in samples DNA extracted from women classified as (1° UI, 2° UI, 1° PCO and 2° PCO). The amplification of the (GSR) gene gave positive results for all the subjected samples, also the amplification revealed polymorphism product of two molecular weight 250 base pair. The result of the sequence analysis was analysed by blast in the National Centre Biotechnology Information (NCBI) online at ([http:// www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and Bio Edit program to detect polymorphism in (GSR) in 2° UI, found 8 mutations between 5 insertion (refers to the insertion of T nucleotide base pairs) showed 99% compatibility and score (217) bits (117) and expect 6e-53 and 3 transversion (refers to the substitution of a Thiamine to adenine ($T \leftrightarrow A$)) showed 98% compatibility score (219) bits (118) and expect 2e-53. The polymorphism in (GSR) 1° PCO and 2° PCO, found 9 insertion mutations (refers to the insertion of T nucleotide base pairs) showed 99% compatibility and score (228) bits (123) and expect 3e-56 and 4 transversion (refers to the substitution of a Thiamine to adenine ($T \leftrightarrow A$)) showed 98% compatibility score (219) bits (118) and expect 2e-53, at the primary PCOS infertility (1°PCO) and secondary PCOS infertility (2°PCO).

The effect of polymorphisms on the MDA level and the distribution of genotype and allele frequency at the women studied groups (1°UI, 2°UI, 1°PCO and 2°PCO). While the effect (Cp, GLH, TSH, Fe, LH, Cu, PRL, TC, TG, VLDL, LDL and SOD) in the allele frequency and (GSR) genotyping Polymorphisms effect in significant regression between studied groups and biochemical parameters.

This study concluded that a disturbance in serum oxidant- antioxidant balance is found in women with (UI) and (PCOS) infertility and suggest a relationship between increased iron and copper concentration and unexplained female infertility. The allele frequency TA polymorphisms in GSR gene are associated with an increased risk of secondary unexplained infertility (2°UI) and PCOS infertility (1°PCO and 2°PCO).



CHAPTER ONE

INTRODUCTION

Infertility is customarily defined as the inability of a sexually active couple with no contraception to achieve natural pregnancy within one year (Kliesch, 2014). The American Society for Reproductive Medicine (ASRM) considers infertility as a disease, which by definition is “any deviation from or interruption of the normal structure or function of any part, organ, or system of the body as manifested by characteristic symptoms and signs; the etiology, pathology, and prognosis may be known or unknown” (Practice Committee of the American Society for Reproductive, 2008).

There are regional and country variability regarding the magnitude of the problem (Agarwal *et al.*, 2015). The American Society for Reproductive Medicine (ASRM) considers infertility as a disease, which by definition is “any deviation from or interruption of the normal structure or function of any part, organ, or system of the body as manifested by characteristic symptoms and signs; the etiology, pathology, and prognosis may be known or unknown” (Practice Committee of the American Society for Reproductive 2008).

Rates in sub Saharan Africa (SSA) range between 12.7 and 16.9% with a midpoint of 14.9% (Agarwal *et al.*, 2015). With respect to the etiology, 30 to 50% of the couple infertility are due to female factors and 20 to 40% related to male factors, 20 to 40% relate to both male and female factors and up to 20% remained unexplained (Sohrabvand *et al.*, 2015)

It has been estimated that 15 % of couples seek medical assistance for infertility, and the origins of the problem seem to be equally distributed between male and female partners (Kliesch, 2014). Taking into account a global perspective and a world population of 7 billion people, Approximately 140 million people (2.2 %) face infertility (US Census Bureau, 2011). Infertility depends at large on the age of the

female partner. As such, the ASRM states that an early evaluation and treatment is warranted after 6 months for women aged 35 years or older (Dorland, 2007). In men, about 8 % seek medical assistance for fertility related problems (CDC, 2014).

In its most updated version (2010) on “the optimal evaluation of the infertile male,” the American Urological Association (AUA) recommends that the initial screening should be done if pregnancy has not occurred within one year of unprotected intercourse, or earlier in cases of known male or female infertility risk factors (American Urological Association, 2014).

Unfortunately, owing to limitations in our understanding of the events that take place during natural conception, and in view of the crude diagnostic tests available to identify potential abnormalities, the cause of infertility is not determined in nearly half of the cases. Moreover, approximately 5 % of couples remain unwillingly childless despite multiple interventions (WHO, 2000; Hamada *et al.*, 2011).

1.1. Infertility – An overview

1.1.1. Definition and Causes

Infertility is defined as the inability to conceive naturally after one year of regular unprotected intercourse. Most of the time, infertility is some degree of subfertility in which 1 in 7 couples need a specialist help to conceive. Subfertility can be either primary or secondary. Primary subfertility is a delay for a couple who have had no previous pregnancies; and, secondary subfertility is a delay for a couple who have conceived previously, although the pregnancy may not have been successful for example, miscarriage, and ectopic pregnancy (Taylor, 2003). Both males and females are equally responsible for the causes. Most of the infertile couples have one of these three major causes including a male factor, ovulatory dysfunction, or tubal-peritoneal disease. According to the Center of Disease Control, the causes of female infertility can be divided into three broad categories including defective ovulation, transport and implantation (Centers for Disease Control and Prevention, 2013).

There are few medical conditions such as polycystic ovarian syndrome (PCOS), endometriosis, bad obstetric history (BOH), peritubal ovarian failure (POF), fibroids, tubal defect etc., which can lead to female infertility in addition to genetic, oxidative stress and environmental factors (Papalou *et al.*, 2016) .

1.1.2. Unexplained Infertility:

Infertility of unknown origin comprises both idiopathic and unexplained infertility (Slobodan *et al.*, 2012). Unexplained infertility is one of the controversial subjects in infertility on which agreement is rarely found among practitioners. It is a term used to define 15% of couples in whom standard investigations including semen analysis, tests of ovulation and tubal patency have failed to detect any gross abnormality

(Schattman *et al.*, 2015). Couples with unexplained infertility suffer from both diminished and delayed fecundity (Glenn *et al.*, 2015). The possible underlying etiologies are defective endometrial receptivity, impaired oocyte quality, peritubal ovarian failure, minimal and mild endometriosis, tubal disease, pelvic adhesions, immunological and endocrinological abnormalities, and oxidative stress (Mallidis *et al.*, 1991) .

A couple is referred for infertility investigation if they are not able to conceive within a year. The diagnosis of unexplained infertility may be frustrating because if there is no explanation for infertility, there is no effective treatment (Glenn *et al.*, 2015). The prognosis is worse if the duration of infertility exceeds 3 years and female partner is > 35 years of age. Treatment has been indicated if the duration is more than 2 years or the female partner is > 35 years of age. Unexplained infertility could also be classified into unexplained primary or secondary infertility (Bhattacharya *et al.*, 2008).

Unexplained female infertility should be a term used when the female reproductive system has been evaluated according to current agreed standards, and no abnormalities are detected (Glenn *et al.*, 2015). Male factor infertility should have been ruled out with at least two normal semen analyses and with no demonstrable physical or endocrine abnormalities (Hamada *et al.*, 2011).

The latest opinion paper by the Practice Committee of the American Society for Reproductive Medicine (ASRM) published in 2012 (Practice Committee of American Society for Reproductive Medicine, 2012). on the diagnostic evaluation of the infertile female, which replaces the 2006 document titled “Optimal evaluation of the infertile female” is a step forward towards understanding female infertility (Nardo and Chouliaras, 2015). Evaluation according to this document should include:

1. Detailed history and clinical examination

2. Assessment of ovulatory function
3. Measuring the ovarian reserve
4. Exclusion of abnormalities of uterine anatomy
5. Confirmation of tubal patency
6. Consideration of peritoneal factors (such as endometriosis).

1.1.3. Polycystic Ovary Syndrome (PCOS):

Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism, chronic anovulation and polycystic ovaries (Dumesic *et al.*, 2015; McCartney and Marshall, 2016). The prevalence of classical forms of PCOS is from 6.9 to 19.9% and is even higher in women with menstrual disorders (17.4-46.4%), hyperandrogenism (72.1-82.0%), and anovulatory infertility (55-91%) (Anderson *et al.*, 2014; Azziz *et al.*, 2009). Clinical symptoms include ovulatory dysfunction, hyperandrogenism, and polycystic ovaries. Metabolic disorders (obesity, insulin resistance, impaired glucose tolerance, type II diabetes mellitus, and dyslipidemia) are typical of 40-85% women with PCOS (Uvarova and Khaschenko, 2013).

PCOS is a risk factor for the development of cardiovascular disorders and morbidity (Anderson *et al.*, 2014). Non-specific biochemical processes in various cell compartments determining organism reactivity and its adaptive potential play a significant role in the pathogenesis of various diseases of reproductive system (Kolesnikova *et al.*, 2012). Lipid peroxidation-antioxidant defense system (LPO—AOD) is considered as one of these regulatory metabolic mechanisms. The balance between LPO and AOD not only reflects, but also determines adaptive capacity of the organism and risk for the development of metabolic disorders (Murri *et al.*, 2013).

1.1.4. Female Infertility and Risk Factors:

Information continues to emerge regarding the important roles played by environmental contaminants and obesity with respect to female reproductive health, and their impact on treatment outcomes associated with this condition (Sutton *et al.*, 2010). Reproductive environmental health is steadily gaining global attention as study after study describe adverse effects of environmental exposures on human reproduction (Caserta *et al.*, 2011). Unexplained female infertility, in particular, appears to have great relevance, as commonly recognized etiologies are ruled out by definition. Many environmental agents are known to display endocrine-disrupting potential, especially on reproductive tract development and function (Sanderson, 2006).

1.1.4.1. Obesity:

Reproductive dysfunctions have been related to both high and low body weight and it may reveal both biological and social influences. Underweight or overweight contributes nearly to one fifth of infertilities (Nelson and Bulun, 2001). Underweight as well as overweight and obesity are linked with an increased risk of anovulatory infertility. Studies have shown that overweight (body mass index [BMI] ≥ 25 kg/m²) or underweight (BMI ≤ 18.5 kg/m²) can contribute to fertility problems. (Rich-Edwards *et al.*, 2002). In a study on Finnish people, it was reported that when compared to women with normal BMI, the probability of having children is less in obese and underweight women (Jokela *et al.*, 2007).

There are many biological and other causes of infertility, which can be treated by medical intervention and lifestyle modification. (Makar *et al.*, 2002). One of such causes that can easily be prevented is deflection from the normal BMI. One of the easiest ways to decide underweight or

overweight is to determine BMI, which is an index of a person's relative "skinniness or heaviness." Various studies concentrated primarily on infertility due to ovulatory disorders and marked the effect of obesity on infertility (Grodstein *et al.*, 1994).

1.1.4.2. Cigarette Smoke:

According to the World Bank, approximately 8 % of women and 39 % of men were identified as tobacco smokers in 2006 (World development indicators, 2010). The highest percentage of female cigarette smokers existed in Europe and Central Asia although 19 and 25 % of United States women and men, respectively, reported active smoking. There are approximately 4000 chemical compounds identified in cigarette smoke, many that are potentially hazardous to reproductive health. Cigarette smoking has long been known to have negative effects on both oocytes and embryos (Neal *et al.*, 2008).

Many epidemiologic studies have demonstrated a clear association between smoking and reduced fecundity (Hull *et al.*, 2000). There is increasing evidence to suggest multiple mechanisms underlying the effect of cigarette smoke. The adverse actions of chemicals in cigarette smoke may act at the levels of ovarian, tubal, and endometrial function. (Benowitz *et al.*, 1983).

1.1.4.3. Toxic Elements:

Another area of emerging interest is the potential effect of toxic elements on female reproductive potential, including arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg). Though nonessential, toxic elements are distributed in ubiquitous fashion, with detectable levels reported for the vast majority of the US population (Centers for Disease Control (CDC) 2009), and investigators have reported accumulation of these agents in female reproductive tissues (Kruger *et al.*, 2012).

Adverse female reproductive effects have been demonstrated rather clearly at high levels of exposure, such as those encountered in the workplace. However, the effects of the lower, or “trace” doses frequently encountered in the environment are more controversial. Epidemiologic studies of populations lacking occupational exposure reported altered sex-steroid hormone economy (Pollack *et al.*, 2011), and have been associated with clinical female infertility, increased time to pregnancy (Louis *et al.*, 2012), and altered oocyte maturation, oocyte fertilization. Elements including As, Cd, Pb, and Hg enter eukaryotic cells and in doing so can disrupt cytoskeletal function, and increase oxidative damage by depletion of protective antioxidant molecules and generation of reactive oxygen species (Bloom, 2012).

1.2. Hormones:

1.2.1. Prolactin (PRL):

Prolactin (PRL) is a polypeptide hormone originally PRL is predominantly produced by the lactotrophic cells of the anterior pituitary gland. However, it is also generated in extra-pituitary sites such as immune, decidual, mammary, epithelial and fat cells (Hugo *et al.*, 2006). Its secretion is under dual regulation by hypothalamic hormones via the pituitary portal circulation. The predominant regulatory signal is the inhibition of prolactin secretion by the neurotransmitter, dopamine, from neurons in the hypothalamus. Evidence suggests that prolactin secretion is regulated by three populations of hypothalamic dopaminergic systems (Grattan and Kokay, 2008).

Several lines of evidence indicate that PRL acts as an autocrine, paracrine, and endocrine progression factor for mammary carcinoma in rodents and human; its actions on reproductive processes represent the largest group of identified functions. Dysovulation is the most common

disorder of the hyperprolactinemia, but the precise mechanisms by which PRL influences the neuroendocrine axis are yet to be deciphered(Bole-Feysot *et al.*, 1998).

A few reports describe the direct effects of prolactin on antioxidative defense. A positive correlation between prolactin and mammary gland superoxide dismutase SOD levels was reported, and SOD expression was induced by prolactin in the rat corpus luteum, while prolactin did not affect the expression of genes encoding superoxide dismutase, catalase, or glutathione peroxidase in the human retinal pigment epithelium cell cultures (Thébault, 2017).

1.2.2. Thyroid Stimulating Hormone (TSH):

Thyroid hormones are secreted by the thyroid gland under the control of the pituitary hormone, thyroid stimulating hormone (TSH) which, in turn, is stimulated by hypothalamic secretion of thyroid releasing hormone. The two active thyroid hormone molecules are L-thyroxine (3, 5, 30, 50 tetraiodothyronine; T4), the major circulating hormone, and 3, 5, 30 triiodothyronine, also known as T3. The hypothalamus-pituitary-thyroid (HPT) axis reacts to a variety of exogenous stimuli capable to modulate T3 and T4, either environmental, such as the day-night rhythm (with a nocturnal peak secretion of TSH in humans, food availability environmental temperature, physical or emotional stress (Machado *et al.*, 2015; Rodrigues *et al.*, 2015), or pathophysiological conditions, such as acute inflammation and critical illness (Fliers *et al.*, 2014).

In older studies, the prevalence of different types of menstrual abnormalities was reported to be about 50% in hyperthyroid women, compared to that in euthyroid women. (Benson and Dailey,1955). Krassas et al. found irregular cycles in 21.5% of thyrotoxic patients, significantly more than the 8.4% in the euthyroid women. Clinical and experimental

studies have suggested a close relationship between Hypothalamo-Pituitary-Ovarian (HPO) axis and Hypothalamo-Pituitary-Thyroid (HPT) axis. The specific thyroid hormone receptors at the ovarian level may regulate the reproductive function (Donfas and Mastorakos, 2000).

Thyroid cells are able to counteract this potentially toxic exposition to reactive oxygen species ROS thanks to a fine tuned antioxidant system, at least until the pro- and anti-oxidant processes are well balanced. However, excess in ROS production and/or inadequate production of antioxidants during aging process may contribute to cause oxidative stress and thyroid damage, including thyroid autoimmune diseases and cancer (Zarkovic, 2012; Vitale *et al.*, 2013).

Additionally, T3 and T4 are known to accelerate basal metabolism and increase oxygen consumption, thus leading to increased reactive oxygen species (ROS) production and oxidative stress. Additionally, T3 and T4 are able to unsaturate membrane phospholipids, leading to membrane damage and mitochondria lipid peroxidation (Gredilla *et al.*, 2001). Such pro-oxidant effects are tissue-dependent, with liver and heart more subject to oxidative stress than spleen and glycolytic muscle fibers. However, T3 and T4 can also affect the cell antioxidant status (iodine compounds act as free radical scavengers able to reduce oxidative damage *in vitro*) (Villanueva *et al.*, 2013).

1.2.3. Luteinizing Hormone and Follicle Stimulating

Hormone:

The gonadotropin hormones Follicle stimulating hormone FSH and Luteinizing Hormone LH are secreted by the pituitary gland. FSH is necessary for the development of the immature ovum, (i.e. the primordial follicle in the ovary), and LH triggers ovulation. The secretion of FSH and LH from the pituitary gland is under the control of Gonadotropin

Releasing Hormone (GnRH) from the hypothalamus, and the hypothalamus in turn is controlled by the levels of the ovarian steroid hormones oestrogen and progesterone in the blood by means a feedback mechanism (Dias *et al.*, 2002).

Follicle stimulating hormone (FSH), a member of the glycoprotein hormone family, plays an essential role in this process (Jennings *et al.*, 2009). Generally, the anterior pituitary gland is the tissue that is responsible for releasing this hormone and granulosa cells in the ovary are the target cells for the numerous actions of FSH (Williams, 2012).

These hormones would mechanistically act via antagonistic pleiotropy—promoting growth and development early in life to advance reproduction but becoming dysregulated and driving senescence once reproductive life span has ended (Leroi *et al.*, 2005).

1.2.4. Female Infertility and Hormones:

Hormones from pituitary gland like TSH, prolactin or growth hormone may act synergistically with FSH and LH to enhance the entry of non-growing follicles into growth phase. In addition to this, thyroid hormones may be necessary for maximum production of both estradiol and progesterone (Wakim *et al.*, 1995).

It is well known that the function of the thyroid hormones includes modulation of carbohydrates, proteins and fat metabolism, gene expression and also sexual and reproductive function and when the thyroid hormone gets out of balance, many body functions are affected.. Thyroid dysfunction in females have been found to be associated with anovulatory cycles, decreased fecundity and increased morbidity during pregnancy (Veeresh *et al.*, 2015).

Hyperprolactinaemia results in the inhibition of gonadotrophin releasing hormone (GnRH) release pulsatility from the hypothalamus and

subsequent inhibition of LH and FSH, leading to the inhibition of ovarian function. High levels of circulating PRL, in physiology and pathological situations, are known to cause infertility (Palm *et al.*, 2001).

Moreover it is associated with menstrual as well as ovulatory dysfunctions like amenorrhoea, oligomenorrhoea, anovulation, inadequate corpus luteal phase and galactorrhoea .

Hypothyroidism can affect fertility due to anovulatory cycles, luteal phase defects, hyperprolactinemia and sex hormone imbalance (Pope *et al.*, 2007). Thyroid dysfunction is a condition known to reduce the likelihood of pregnancy and to adversely affect pregnancy outcome.

Abnormal patterns of gonadotropin secretion, including increased serum luteinizing hormone (LH) concentrations, low-normal follicle-stimulating hormone (FSH) levels, and increased LH/FSH ratios have long been recognized as common characteristics of women with PCOS (Fritz and Speroff , 2011).

Studies in women with PCOS (who characteristically have more frequent LH pulses, higher LH levels and higher LH/FSH ratios than normal women with regular menstrual cycles) have revealed no effect of a 12 h hyperinsulinaemic euglycaemic clamp on LH secretion (Moret *et al.*, 2009). Furthermore, LH responses to Gonadotropin Releasing Hormone GnRH were not influenced by insulin administration, suggesting that insulin resistance and compensatory hyperinsulinaemia are not the direct causes of the abnormal hypothalamic GnRH pulse generator activities in PCOS patients (Comninou *et al.*, 2014).

1.3. Oxidative Stress:

1.3.1. Reactive Oxygen Species (ROS):

The generation of reactive oxygen species (ROS) is a normal feature of basal aerobic metabolism that supports life. They are active derivatives produced during the intermediate steps of oxygen reduction, which are catalyzed by such as iron and copper atoms molecules within cells, and are also necessary for the physiological processes of reproduction: ovulation, capacitation and corpus luteum formation and function (Shkolnik *et al.*, 2011; Vu *et al.*, 2012).

Some reactive oxygen species ROS also act as messengers in cell signalling as deregulation of the redox status will disrupt signalling mechanisms. This has been shown in the case of endometriosis, in which ROS are involved in its progression (Yves *et al.*, 2016). Therefore, ROS play positive physiological roles, through involvement in signal transduction pathways. At the present time, more than 10 redox-sensitive transcription factors have been identified, affecting various kinases, cell growth and differentiation, either directly or indirectly, as GSH regulates and activates several transcription factors, such as AP-1, NF- κ B and Sp-1. (Sun *et al.*, 2016).

Biological systems contain an abundant amount of $O_2^{\bullet -}$ free radicals are often generated from O_2 and partially from normal metabolic processes in the body. They are unstable and highly reactive due to unpaired electrons that are capable of initiating an uncontrolled cascade of chain reactions, resulting in cellular damage and disease (Sikka, 2004).

The three major types of reactive oxygen species ROS are superoxide ($O^{\bullet -}_2$), which are formed when electrons leak from the electron transport chain; hydrogen peroxide (H_2O_2), resulting from the dismutation of superoxide or directly from the action of oxidase enzymes, and hydroxyl

(HO•), a highly reactive species that can modify purines and pyrimidines and cause strand breaks that result in DNA damage. ROS have been implicated in more than 100 diseases (Madamanchi *et al.*, 2005). They have a physiological role in the female reproductive tract. Numerous animal and human studies have demonstrated the presence of ROS in the female reproductive tract: ovaries, fallopian tubes and embryos (Guerin *et al.*, 2001).

The deleterious effects of ROS. are summarized as follows:

1. Opening of ion channels: Elevated levels of ROS release Ca^{+2} from endoplasmic reticulum resulting in mitochondrial permeability, so the mitochondrial membrane potential becomes unstable and ATP production ceases (Behl Pandey, 2002).
2. Lipid peroxidation: It occurs in polyunsaturated fatty acid side chains. These chains react with O_2 creating the peroxy radical, which can obtain H^+ from another fatty acid, creating a continuous reaction.
3. Protein modifications: Amino acids are targets for oxidative damage. Direct oxidation of side chains can lead to the formation of carbonyl groups.
4. DNA oxidation: Mitochondrial DNA is susceptible to ROS due to the presence of O_2^- in the electron transport chain, lack of histone protection, and absence of repair mechanisms (Gupta *et al.*, 2009).

Mechanisms of oxidative stress-induced cell damage shown in Figure (1.1).

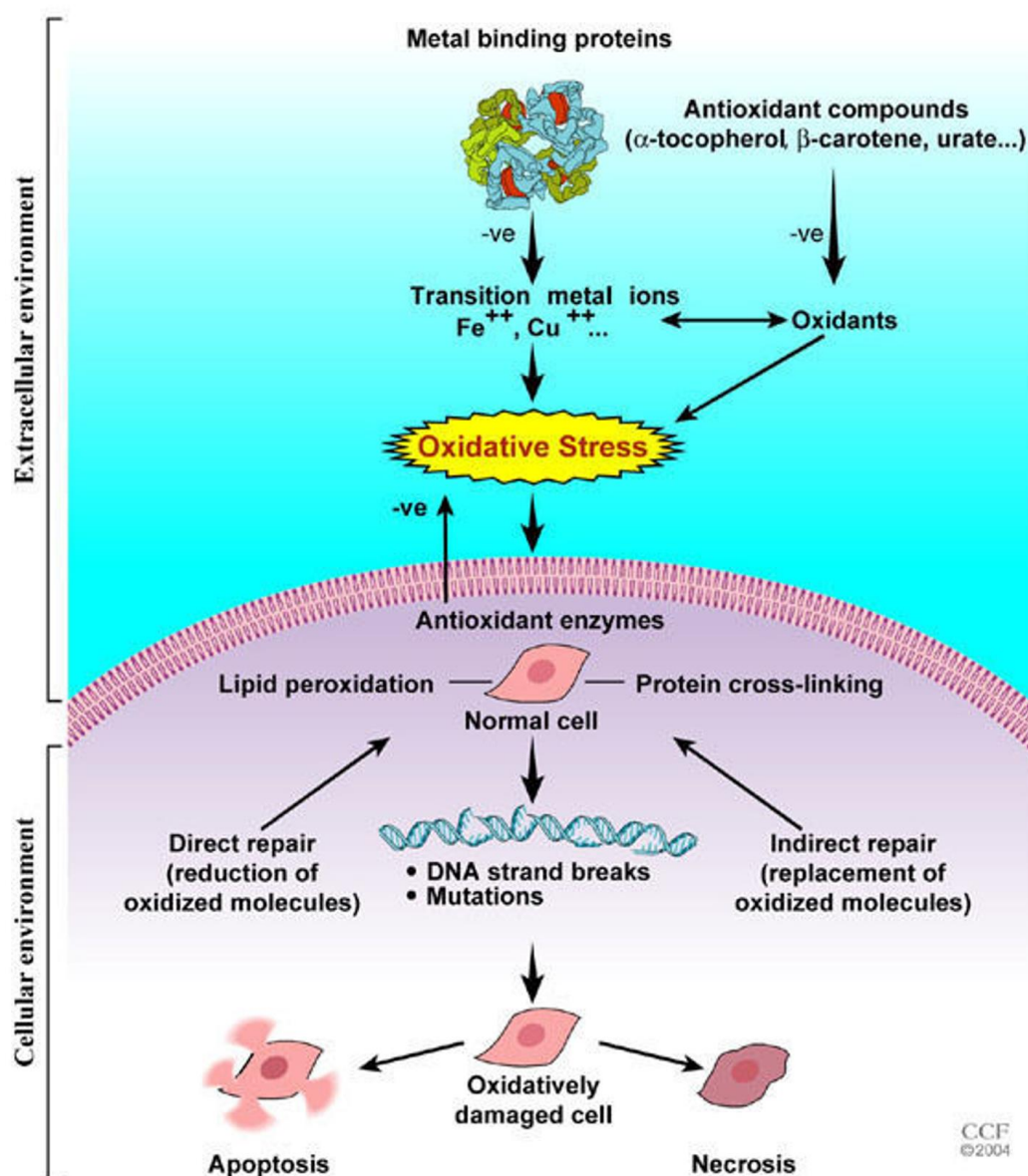


Figure (1.1): Mechanisms of oxidative stress-induced cell damage
(Agarwal *et al.*, 2005)

ROS may react with other molecules to disrupt many cellular components and processes. ROS target the pathway and also act as second messengers in some reactions. ROS are known to effect ovulation and implantation (Al-Gubory *et al.*, 2012). The presence of ROS and antioxidants in the female reproductive tract has been demonstrated by various methodologies in animal and human studies. A number of

oxidative stress biomarkers have been investigated including superoxide dismutase, glutathione peroxidase, lipid peroxides, thiobarbituric acid reactive substances, oxidative DNA adducts, follicular fluid, nitric oxide NO and total antioxidant capacity. Inorganic markers include excessive levels of iron (Fe) and Copper (Cu). A high Cu/Zn ratio is a good indicator of oxidative stress (Brack *et al.*, 2013; Brack *et al.*, 2016).

1.3.2. Antioxidant System:

Under normal conditions, scavenging molecules known as antioxidants convert ROS to H₂O to prevent overproduction of reactive oxygen species (ROS.). There are two types of antioxidants in the human body: enzymatic antioxidants and non-enzymatic antioxidants (Pierce *et al.*, 2004). Enzymatic antioxidants are also known as natural antioxidants; they neutralize excessive ROS and prevent it from damaging the cellular structure. Enzymatic antioxidants are composed of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, which also causes reduction of hydrogen peroxide to water and alcohol (Agarwal *et al.*, 2005).

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements (Van *et al.*, 2002). The body's complex antioxidant system is influenced by dietary intake of antioxidant vitamins and minerals such as vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, glutathione, beta carotene, and carotene (Agarwal *et al.*, 2003).

Enzymatic antioxidant defenses have been found in mammalian embryos and oocytes and nonenzymatic defenses in tubal and follicular fluids. Increased levels of antioxidants have been shown in normal pregnancy (Gupta *et al.*, 2007), whereas loss of antioxidant defenses have been observed in patients with recurrent abortion as a result of their

increased consumption. The lower antioxidant levels could aggravate pro-oxidant injury in endothelial cells, altering prostacyclin–thromboxane balance and may contribute to preeclampsia or abortion (Wang *et al.*, 1991).

1.3.2.1. Glutathione (GSH):

GSH is the most widespread low-molecular-weight thiol in nature. (DeLeve and Kaplowitz 1991). Almost 90% of GSH is in the cytosol, about 10% in mitochondria, and small amounts also in the endoplasmic reticulum (Lu, 1999). More than 98% of intracellular GSH is its reduced form; the rest is glutathione disulfide (GSSG), mixed disulfides (mainly GSS proteins) and thioethers. GSH synthesis takes place in the cytosol. It is a two-step reaction, catalysed by γ -glutamyl cysteine synthetase and glutathione synthetase, which takes place mainly in liver (DeLeve and Kaplowitz, 1991; Lu, 1999).

Functionally, glutathione is a biologically dispersed, pivotal antioxidant counterpart which is categorized under the thiol family, and directly interacts with H_2O_2 to reduce it to biochemically stable forms of H_2O and O_2 (Presnell *et al.*, 2013). Deeper insights into the biochemical mechanism show that the reduced form of GSH reacts with H_2O_2 via an enzymatic process catalyzed by glutathione peroxidase. Once the reaction is in progress, the available GSH is converted into the oxidized form of glutathione (GSSG) (Toppo *et al.*, 2009).

The regeneration of GSH from GSSG is controlled by a supplementary enzymatic reaction which is catalyzed by the enzyme glutathione reductase, also known as glutathione reductase (GR) (Jones, 2002). As GR is the regulating factor of the aforementioned reaction, it helps to maintain the internal GSH/GSSG ratio at an optimal level, which is an indicator of oxidative stress as well as changes in redox signaling and

control (Zhang, 2012). In a stepwise manner, the mechanism of the corresponding catalytic reaction goes through two distinguishable phases: the first portion being reduction and second portion oxidation (Jones, 2002), as shown in Figure (1.2).

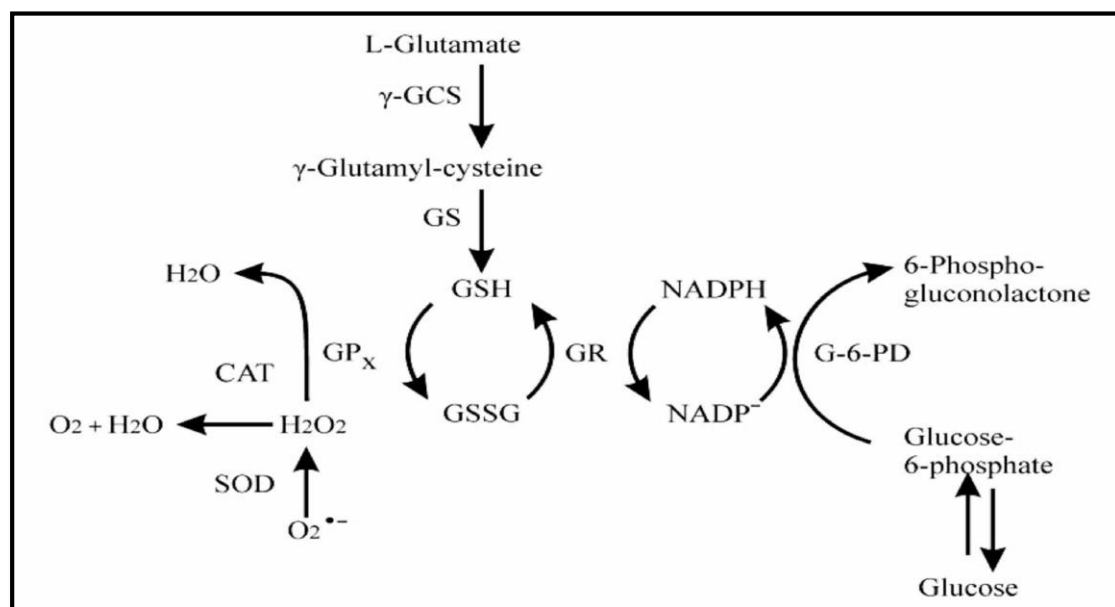


Figure (1.2): mechanism of the GSH as scavenger free radicals through direct chemical reactions (Kand'ár, 2015).

Therefore, continuity of these processes on the activity of superoxide dismutase (SOD) is important. Important antioxidant functions of GSH also include α -tocopherol and ascorbate regeneration and protection of a thiol group of proteins (Kand'ár, 2015), various factors influencing the GSH concentration in plasma and whole blood such as physical exercise, cold exposure, diabetes mellitus, cardiovascular disease, cancer and chronic diseases (Rossi *et al.*, 2002).

The erythrocytes are relatively rich in GSH (Rizzo *et al.*, 2012). GSH helps maintain the normal structure of membranes, prevents denaturation of haemoglobin, keeps the ions in the reduced state and is able to reduce methaemoglobin (Fe^{3+}) to haemoglobin (Fe^{2+}). Nicotinamide adenine

dinucleotide phosphate NADPH is required to maintain GSH in the reduced state (DeLeve and Kaplowitz, 1991).

Glutathione is present in the oocyte and tubal fluid and has a role in improving the development of the zygote beyond the 2-cell block to the morula or the blastocyst stage (de Matos and Furnus, 2000). The glutathione oxidation–reduction ratio is a good marker of both oxidative stress and antioxidant status (Brack *et al.*, 2013). The question of gender-related resistance to oxidative stress has always been a subject of debate. Are there differences between men and women? This is no longer open to question: men and women have a different antioxidant status, with sensitivity and response to oxidative stress being better in women (Brack *et al.*, 2016).

Conversely, it was also seen that glutathione was decreased in the peritoneal fluid of patients with unexplained infertility.. Hence, one theory is that underlying cause of unexplained infertility is the disruption of the delicate balance that exists between ROS and antioxidant levels (Gupta *et al.*, 2014). Selenium is part of the selenoenzyme glutathione peroxidase and protects the integrity of membrane structures by enzymatically degrading endogenous and exogenous hydrogen peroxide and lipid peroxides (Paszkowski *et al.*, 1995).

1.3.2.2. Superoxide Dismutase (SOD):

Superoxide dismutase SOD (EC1.5.1.1) enzymes are a family of metalloenzymes, found in virtually every oxygenbased organism, and their major function is to catalyze the dismutation of $O_2^{\cdot -}$ to H_2O_2 (Zelko *et al.*, 2002), as described earlier. This reaction is generally considered to be the body's primary antioxidant defense because it prevents further generation of free radicals. Depending on the metal ion cofactor (copper, zinc or manganese), three isoforms of SOD are recognized to be

important, which are also distributed differently in the body (Miao and Clair, 2009). In humans, manganese (Mn) SOD is present in the mitochondrion, copper-zinc (Cu/Zn) SOD is present in the cytosol, while a third form of SOD in extracellular fluids also contains copper-zinc (Cu/Zn) in its active sites (Zelko *et al.*, 2002, Nozik-Grayck *et al.*, 2005). In humans, SOD occurs in high concentrations in brain, liver, heart, erythrocytes, kidney and spleen (Zelko *et al.*, 2002; Aprioku, 2013).

Corpus luteum is produced after ovulation and ROS are also produced in the corpus luteum. Cu, Zn-SOD decreases and ROS increases during the regression of corpus luteum (Verit, 2015). This activity parallels the change in progesterone levels. Complete disruption of the corpus luteum causes a substantial decrease of Mn-SOD in the regressed cell and cell death is imminent (Shkolnik *et al.*, 2011). Cu, Zn-SOD is related to progesterone production and Mn-SOD protects luteal cells from oxidative stress (Behrman *et al.*, 2001).

1.3.2.3. Glutathione Disulfide Reductase (GSR):

Genetic factors play a crucial role in female infertility. Therefore, there is an expanding interest in the role of genetic factors like glutathione disulfide reductase (GSR) and manganese superoxide dismutase (MnSOD) polymorphic variants in female infertility (Faure *et al.*, 2014). The glutathione (GSH) family of enzymes includes glutathione peroxidase GPx, glutathioneS-transferase GST, and glutathione reductase GSR. GPx uses the reduced form of GSH as an H⁺ donor to degrade peroxides. Depletion of GSH results in DNA damage and increased H₂O₂ concentrations; as such, GSH is an essential antioxidant. During the reduction of H₂O₂ to H₂O and O₂, GSH is oxidized to GSSG by GPx. Glutathione reductase participates in the reverse reaction, and utilizes the

transfer of a donor proton from NADPH to GSSG, thus recycling GSH (Perkins, 2006).

Glutathione reductase (GR) catalyzing the Reaction ($\text{GSSG} + \text{NADPH} + \text{H}^+ \longrightarrow 2\text{GSH} + \text{NADP}^+$) is a key enzyme in this system, because it maintains millimolar concentrations of reduced glutathione (GSH), rendering GSH the most abundant reductant in human cells. GR is a homodimeric flavoprotein with a subunit *Mr* of 52.4 kDa. Its 2 identical redox active sites are formed by residues from both subunits implying that monomeric GR is not active (Ho *et al.*, 1998). Human GR is encoded by a single gene (*GSR*, GeneID: 2936, MIM 138300), located on chromosome 8p21.1 and consisting of 13 exons spanning 50 kb (Kelner and Montoya, 2000). The gene contains 2 in-frame start codons that are thought to initiate the synthesis of mitochondrial and cytosolic GR (Outten and Culotta, 2004).

Since the holoenzyme consists of apogluthathione reductase (apoGR) and FAD as a prosthetic group, the lack or reduction of GR activity can have 2 causes. First, due to inherited mutations, the GR protein can be absent or exhibit low catalytic activity. Second, acquired FAD deficiency due to low amounts of riboflavin (vitamin B2) in the diet (or failure to convert it sufficiently to FAD) may result in inactive apo GR. In the latter case, GR activity can be restored by riboflavin administration in vivo or FAD addition in vitro (Loos *et al.*, 1976).

Whereas inherited glutathione reductase deficiency is rare, FAD deficiency is common in malnourished populations. The clinical symptoms of GR deficiency include reduced lifespan of red blood cells (RBCs), cataract, and favism (Nanne *et al.*, 2007). Functionally, *GSR* has an indirect effect on a diverse array of biological processes via glutathione, despite its primary role as a crucial player in glutathione

metabolism. Human glutathione has been widely studied and identified to be present in the detoxification mechanisms of xenobiotics (Sipes *et al.*, 1986), as a cofactor in isomerization reactions (Pettersson and Mannervik, 2001), as a storing and transporting agent of essential cysteine amino acid residues (Volodymyr, 2012) and so forth. Moreover, a study reveals that GSH levels interfere both with cell proliferation and the regulatory mechanisms of apoptosis (Day and Suzuki, 2005).

Under septic conditions, H_2O_2 exceeds basal levels as a response to pathogenic infection. Expression of the GSR gene is a prerequisite to reverting excessive H_2O_2 to baseline levels as the GSR enzyme plays a predominant role in the process of H_2O_2 elimination. In addition, in mammalian studies, it is a well-accepted fact that GSR plays a fundamental role in host defense against bacterial infections (Yan *et al.*, 2013).

1.3.2.4. Ceruloplasmin:

Ceruloplasmin is encoded by a single gene. The main Cp form (in plasma, cerebrospinal fluid and milk) contains 1046 amino acids (Linder, 1991). Plasma Cp is mainly produced by hepatocytes. Cells in several other secretory organs also produce Cp, including kidney, mammary gland, placenta, and the brain (Donley *et al.*, 2002; Linder, 2010), also macrophages and mononuclear cells in the blood during inflammation. (Marques *et al.*, 2012).

All the established and documented functions of Cp are based on the fact that it contains and carries copper i.e. the known functions are of the holo form (Zaitseva *et al.*, 1996). Whether the almost equally abundant apo form in the plasma plays some kind of independent role in mammalian metabolism (or copper metabolism) has not been explored (Linder, 2016).

Cp was shown to inhibit a variety of oxidative reactions, involving peroxide and superoxide, including the Fenton reaction that forms $\cdot\text{OH}$ radicals from H_2O_2 , and dismutation of superoxide (Linder, 1991).

Most of the roles of holoCp are enzymatic, involving the absorption and transfer of electrons from various substrates to oxygen. Several classes of substrates have distinct binding sites on the molecule. (Zaitsev *et al.*, 1999). Cp is best known for its ability to oxidize Fe^{2+} to Fe^{3+} (ferrooxidation), and it is the principal ferrooxidase in the blood plasma (Harris *et al.*, 1999). It is generally accepted that the ability of Cp to oxidize iron facilitates the release of this metal from cells and allows iron binding to transferrin in extracellular fluid and blood (Vashchenko and MacGillivray, 2013).

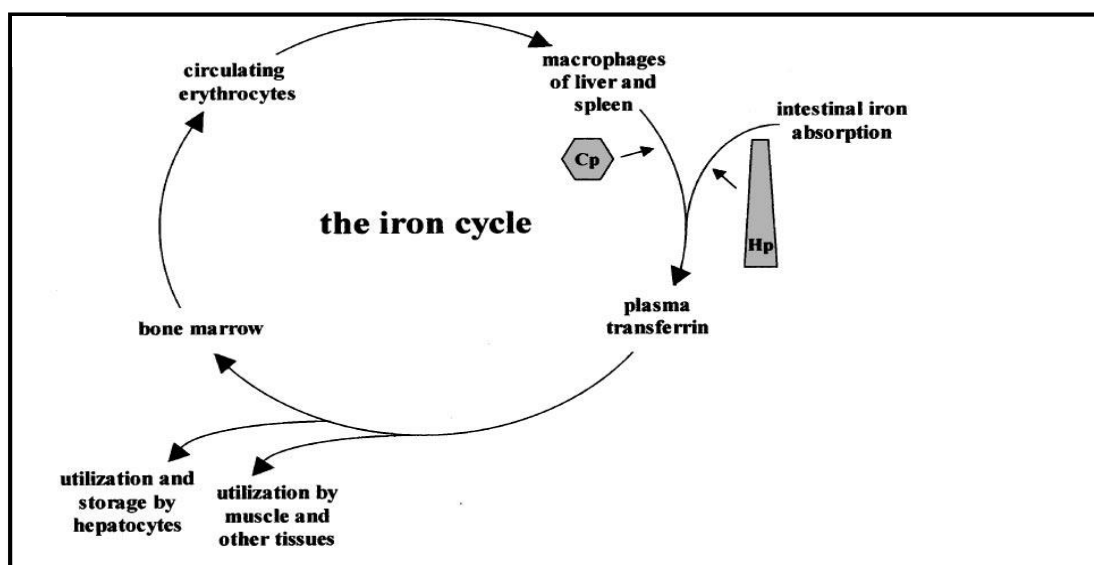


Figure (1.3): Role of the multicopper oxidases in the iron cycle
(Hellman and Gitlin, 2002).

Cp is one of the main extracellular radical scavengers. Its ability to dampen or nullify the effects of reactive oxygen species was demonstrated long ago with purified Cp in vitro principally by the groups of Goldstein and Gutteridge (Goldstein and Charo, 1981; Gutteridge *et al.*, 1985). Indeed, patients not expressing Cp (aceruloplasminemia) have

increased lipid peroxidation in portions of the brain associated with iron accumulation (Yoshida *et al.*, 2000; Miyajima *et al.*, 2002). However, a second reason for the resulting damage in aceruloplasminemia would be due to lack of the antioxidant function of Cp to deal with the radicals and prevent cellular damage (Grolez *et al.*, 2015).

1.3.3. Oxidative Stress and the Ovarian Functions:

Oxidative stress is important in ovarian germ cell and stromal cell physiology. A cohort of oocytes begins to grow and develop in the ovary each month (Gupta *et al.*, 2009). Hypoxia of the granulosa cells is a normal event during the growth of ovarian follicles (Tropea *et al.*, 2006). Hypoxic or anoxic conditions reduce ROS and increase antioxidant activity (Basini *et al.*, 2004). It has been found that cyclical ROS production and diminished antioxidant activity may contribute to oophoritis associated with autoimmune premature ovarian failure. Meiosis II is promoted by antioxidants (Behrman *et al.*, 2001).

Granulosa and luteal cells respond negatively to ROS and affect Meiosis II progression, leading to diminished gonadotropin, and antisteriodogenic actions, DNA damage, and inhibited ATP progression in humans (Behrman *et al.*, 2001; Verit, 2015). Glutathione, an antioxidant has been identified as critical for oocyte maturation, (Eppig, 1996; Ikeda *et al.*, 2005).

The steroid production in the leading follicle causes an increase in cytochrome P450 enzyme (Hanukoglu, 2006). Overexposure of the ovary to H₂O₂ causes the LH receptor to uncouple from adenylate cyclase, impairing protein synthesis and cholesterol utilization by mitochondrial P450 side chain cleavage, most likely because of impaired production of steroidogenic acute regulatory protein (StAR) (Behrman *et al.*, 2001, Verit, 2015). StAR is responsible for moving cholesterol to the inner

mitochondrial membrane where P450scc converts cholesterol to pregnenolone (Behrman and Aten, 1991; Hanukoglu, 2006).

Oxidative stress is important in ovulation (Rude *et al.*, 2009). LH surge is essential for ovulation that is triggered by oxidative stress (Shkolnik *et al.*, 2011). Follicular ROS promote apoptosis and glutathione and FSH counterbalance its action. Estrogen increases in response to FSH, triggering the generation of catalase in the dominant follicle, thus avoiding apoptosis (Devine *et al.*, 2012).

Corpus luteum is produced after ovulation and ROS are also produced in the corpus luteum. Cu, Zn-SOD decreases and ROS increases during the regression of corpus luteum (Agarwal, 2005). This activity parallels the change in progesterone levels. Complete disruption of the corpus luteum causes a substantial decrease of Mn-SOD in the regressed cell and cell death is imminent (Shkolnik *et al.*, 2011).

1.3.4. Oxidative Stress and Aging Oocyte:

ROS increase with age that may contribute to follicular atresia and decline in the number and quality of oocytes (Wiener-Megnazi *et al.*, 2004; Tatone *et al.*, 2008). It has been reported that prolonged exposure of aged oocytes to ROS negatively affects calcium hemostasis and impairs Ca^{+2} oscillation-dependent signaling and causes decline in oocyte developmental ability (Takahashi *et al.*, 2003). Oxidative stress damages telomeres and accelerates telomere shortening (Richter *et al.*, 2007). Telomere shortening and dysfunction may lead to defects in meiosis, fertilization, and embryo development (Liu *et al.*, 2004). In another study, it has been found that follicular fluid catalase and glutathione transferase were lower in older women compared with young ones (Carbone *et al.*, 2003). Studies have demonstrated that N-acetyl-L-cysteine and vitamin E protect the ovary from aging (Yeh *et al.*, 2005; Liu *et al.*, 2012).

1.3.5. Oxidative Stress and Female Infertility:

Excessive ROS production, however, may overpower the body's natural antioxidant defence system, creating an environment unsuitable for normal female physiological reactions (Agarwal *et al.*, 2012). This, in turn, can lead to a number of reproductive diseases including endometriosis, polycystic ovary syndrome (PCOS) and unexplained infertility. As shown in figure (1.4).

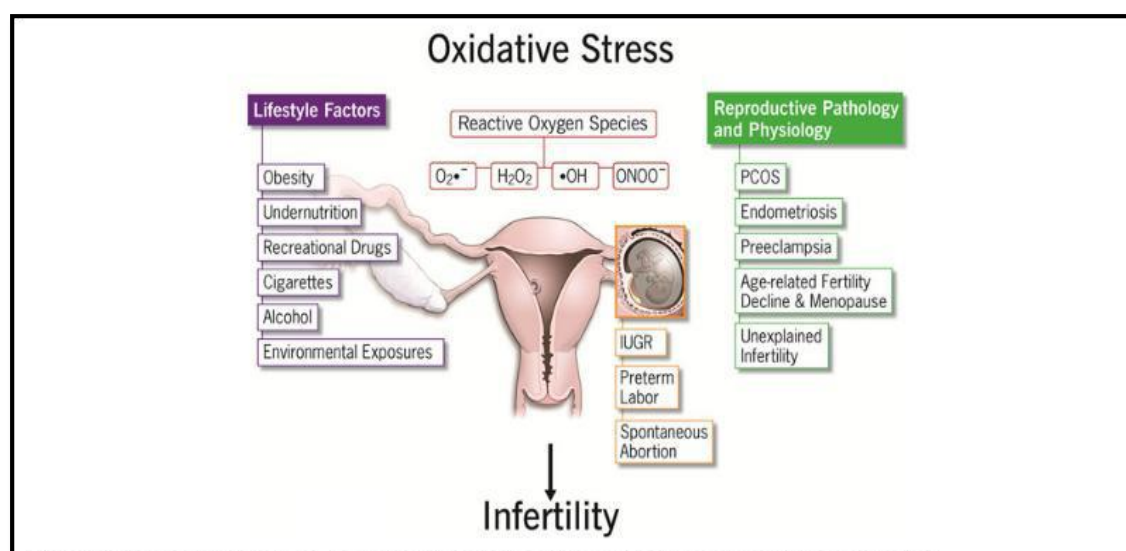


Figure (1.4): Factors contributing to the development of oxidative stress and their impacts on female reproduction. (Agarwal *et al.*, 2012).

1.3.5. Oxidative Stress and Unexplained Infertility:

Oxidative stress has been implicated in the pathophysiology of the disease. Lipid peroxidation marker, Malondialdehyde MDA, was increased and total antioxidant capacity TAC was decreased in the peritoneal fluid of women with unexplained infertility (Polak *et al.*, 2001). It has been hypothesized that peritoneal fluid diffuses into the fallopian tube where it may cause damage to sperm. Folate is a B9 vitamin that plays a role in amino acid metabolism, and the methylation of proteins, lipids, and nucleic acids (Verit, 2015). Acquired or hereditary folate deficiency results in homocysteine accumulation. Polymorphisms

in folate-metabolizing pathways of genes may be responsible for unexplained infertility in this group (Altmäe *et al.*, 2010) .

Hyperhomocysteinemia may activate apoptosis leading to follicular atresia. Pregnancy and implantation rates were lower and abortion rates were higher in women elevated homocysteine. It disturbs the endometrium and contributes to poor oocyte quality (Altmäe *et al.*, 2010). Endothelial nitric oxide synthase eNOS mRNA in the endometrium of the women with unexplained infertility was significantly higher compared with controls. Its increased expression suggests the detrimental effect of NO in endometrial receptivity and implantation (Najafi *et al.*, 2012).

The excess of free radicals in the female reproductive system can lead to reproductive pathologies of the genital tract (Asplund *et al.*, 2007). Studies have found definite links midst the onset of OS and the development of endometriosis, tubal factor infertility, polycystic ovarian disease, unexplained infertility and recurrent pregnancy loss (Harrison *et al.*, 2011). Oxidative stress in the female reproductive tract can also severely affect oogenesis and embryo development. These aforementioned pathologies are attributed to various mechanisms including lipid peroxidation, DNA damage, inhibition of protein synthesis, mitochondrial alterations, and depletion of ATP (Thomson *et al.*, 2008).

Increased Reactive Oxygen Species (ROS) in patients with unexplained infertility suggest reduced levels of antioxidants such as vitamin E and GSH would reduce (ROS) scavenging ability and prevent the neutralization of toxic ROS effects; however, the use of antioxidants in women with unexplained infertility is unclear. Studies are needed to explore the efficacy of antioxidant therapy in these patients (Verit, 2015). Selenium (Se) and glutathione peroxidase (GPx) levels were found to be the lowest in patients with unexplained infertility. Consequently reduced

amount of antioxidants in ovarian follicle could be a reason for unexplained infertility (Gupta *et al.*, 2014).

1.3.6. Oxidative Stress and PCOS:

Oxidative stress generally known to be present in PCOS women regardless of whether they are lean or have metabolic abnormalities, has been documented in infertile PCOS women (Sabuncu *et al.*, 2001). The profound factors in PCOS that increases OS are Insulin resistance IR and hyperandrogenemia HG, however, non-obese PCOS patients without IR were also reported to have elevated oxidant status (Verit and Erel, 2008). Lipid peroxidation process revealing malonyldialdehyde (MDA) is accepted to reflect OS (Karadeniz *et al.*, 2008), while GSH and SOD, an antioxidant enzymes serves as a defensive mechanism of the body. Intracellular and cell wall damage due to increased ROS leads to an increase in MDA levels (Hyderali and Mala, 2015). While SOD defends the body against free oxygen radicals and balances the increased oxidative load levels (Karabulut *et al.*, 2012).

Indeed, a reduction in the GSH/ GSSG ratio and GSH levels occurs due to the increase in ROS production and a decrease in oxygen consumption which results in impaired mitochondrial function in PCOS women (Dincer *et al.*, 2005).

Furthermore, infertility in PCOS patients was related to the effect of follicular fluid's oxidative stress levels on the meiotic spindle formation in the oocyte (Gupta *et al.*, 2014). Other studies indicate that elevated ROS in the follicular fluid tends to limit the fertilization potential of oocytes, through disruption of meiotic spindle formation (Chattopadhyay *et al.*, 2010).

Hence, the status of antioxidants in polycystic ovarian syndrome (PCOS) remains inconclusive; However, the prevalence of oxidative stress in PCOS is definite (Lee *et al.*, 2010). Due to the prevalence of pro-

oxidant status in PCOS women (Fenkci *et al.*, 2003), studies identified the ameliorative effects of antioxidant supplementation (Sekhon *et al.*, 2010). Antioxidant supplementation enhanced the activity of antioxidant enzymes such as SOD and GPx, while it decreased the levels of biomarkers of OS such as MDA and lipid peroxides (Mier-Cabrera *et al.*, 2009).

1.4. Lipids Profile:

The term lipid is used to identify a heterogeneous group of compounds, which include oils, fats, waxes, and other related compounds. Lipids are classified into simple lipids, compound lipids, and derived lipids, examples of which are TG, lipoproteins, and cholesterol, respectively (Mayes, 2000). Approximately 98% of dietary lipids exist as TG, and about 90% of the total body fat is stored as TG in the adipose cells of subcutaneous tissue (McArdle *et al.*, 2001).

The lipid structure of erythrocyte membrane differs greatly from those of other cells because erythrocytes lack lipoprotein receptors and de novo cholesterol synthesis (Tziakas *et al.*, 2010). Although erythrocyte membrane cholesterol (EMC) content is correlated with plasma lipid composition, the mechanism concerning a possible interaction of EMC with the lipoprotein subfractions is not yet clear. (Vaya *et al.*, 2008; Tziakas *et al.*, 2008 , 2012).

Lipids provide structural integrity for all tissues, and they are major source of energy for metabolism, physical activity, and storage. Lipids are necessary precursors for hormones (Bashorik *et al.*, 2001). Steroid hormones are derived from cholesterol. Reproduction, growth, development, and neural functions, all depend on appropriate supplies of lipids (Dupont, 1998). Due to their hydrocarbon nature, most lipids are not very soluble in water. To be transported in the circulation, they need to be packaged into lipoprotein complexes in the blood (Marinetti, 1991).

Lipoproteins are large globular particles; they contain an oily core of predominantly TG and cholesterol esters (CE) surrounded by an outer polar membrane consisting of phospholipids, free cholesterol, and Apo-proteins. The polar hydrophilic groups on the outer surface of the membrane confer water solubility on the lipoproteins (Marinetti, 1991). Lipoproteins can be classified on the basis of their density, molecular size, and electrophoretic mobility. Using all these methods, four distinct classes of lipoproteins have been identified that are important physiologically and clinically (Mayes, 2000). Lipoproteins play a major role in the atherosclerotic processes leading to cardiovascular events (Matthews *et al.*, 1989).

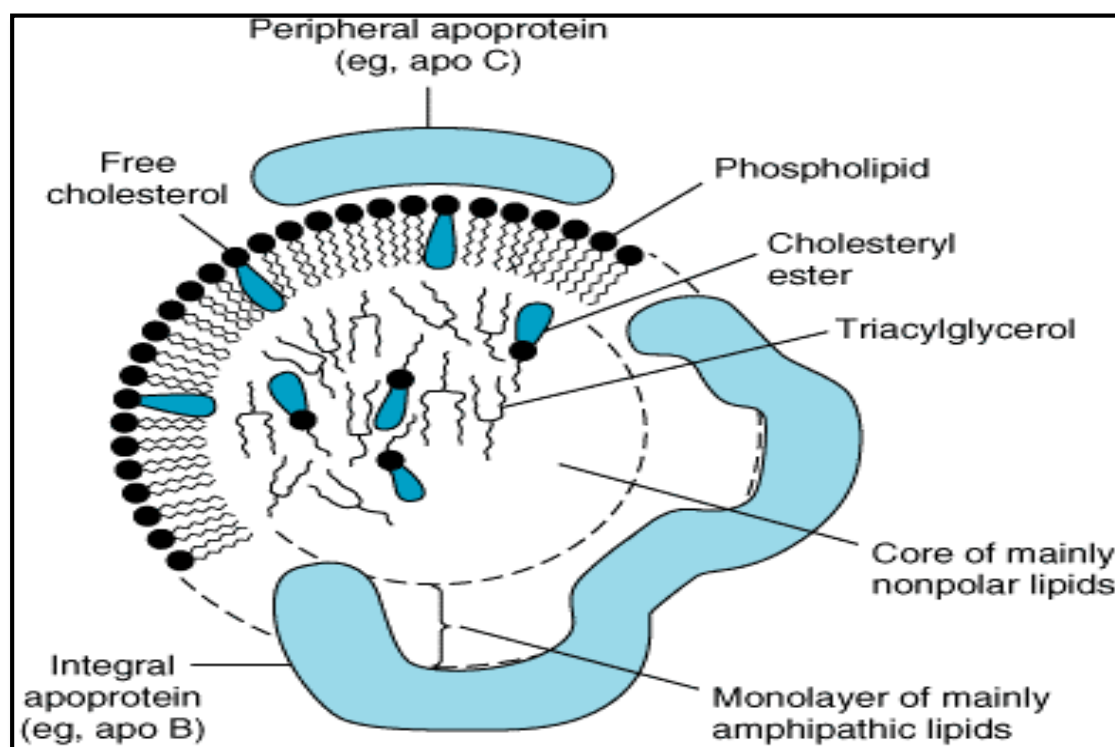


Figure (1.3): Generalized Structure of Lipoprotein. with small amount of cholesteryl ester and triacylglycerol found in the surface layer and little free cholesterol in the core. (Robert *et al.*, 2003).

1.4.1. Low-Density Lipoprotein Cholesterol (LDL):

Most low density lipoprotein cholesterol LDL is a product of VLDL catabolism. Low-density lipoprotein cholesterol is made up of 21% protein and is a cholesterol-enriched and TG depleted particle. Low-density lipoprotein cholesterol holds 50-70% of the total blood cholesterol. LDL serves to supply cholesterol to many cells of the body where it is used in the synthesis of cell membranes and steroid hormones (Marinetti, 1991). The major Apo lipoprotein for LDL is Apo B-100; it is inherited from the VLDL. LDL carries cholesterol in the blood and deposits it in the body tissues and walls of blood vessels (Griffin and Packard, 1994).

1.4.2. High-Density Lipoprotein Cholesterol (HDL):

HDL collects cholesterol from the body's tissues and brings it back to the liver. Sometimes referred to as the "good cholesterol" lipoprotein. This lipoprotein is thought to be developed after ingestion of a large amount of cholesterol. Both the liver and the intestine are involved in the production of HDL. Little is known about the site of HDL catabolism; however the liver and kidney may be involved. Also HDL serves as a potent endogenous inhibitor of inflammation, platelet adhesion, and LDL oxidation (Navab *et al.*, 2001).

1.4.3. Triglycerides:

Triglycerides are tri esters consisting of glycerol molecule coupled to three fatty acid residues (R) of varying carbon chain lengths and degrees of saturation. The two terminal carbon in glycerol are chemically equivalent (Gerhard *et al.*, 1998). Triglycerides in plasma are derived from fats eaten in food or made in the body from other energy sources like carbohydrates. Excess triglycerides in plasma are called

hypertriglyceridemia. It is linked to the occurrence of coronary artery diseases in some people. Increased fish oil intake may be substantially lower an individual's triglycerides (Terres *et al.*, 1991).

1.4.4. Very Low Density Lipoprotein (VLDL):

VLDL is formed by the combination of cholesterol, triglycerides and apoprotein in the liver. These lipoprotein particles are smaller than chylomicrons (Griffin, 1994). It is assembled in the liver from cholesterol and apolipoproteins. It is converted in the bloodstream to low density lipoprotein (LDL). VLDL particles have a diameter of 30-80 nm. VLDL transports endogenous products where chylomicrons transport exogenous (dietary) products. VLDL transports endogenous triglycerides, phospholipids, cholesterol and cholesterol esters (Attman *et al.*, 1993).

1.4.5. Female Infertility and Lipid:

Human female infertility, which in many cases is of unknown etiology, is a significant medical problem (te Velde and Cohlen, 1999). The influence of abnormal lipoprotein metabolism on female infertility has not been thoroughly explored, despite observations suggesting a potential role for plasma lipoproteins, especially HDL (Handwerger *et al.*, 1999). Lipoproteins transport between tissues a number of lipids (e.g., cholesterol, steroid hormones, and vitamin E) that either directly, or indirectly through their metabolic products, play key roles in fertility (Farese and Herz, 1998).

In many species, including humans, the only lipoprotein detected in substantial amounts in the follicular fluid surrounding the developing oocyte in the ovary is HDL. HDL may deliver critical lipid nutrients to either the follicular cumulus cells or the oocytes for membrane synthesis, local steroid hormone production, or other processes essential for normal oocyte maturation. It might also have a role in cholesterol efflux from the

oocyte/cumulus cells, thus participating in the maintenance of cellular cholesterol balance. Therefore, abnormalities in HDL metabolism affecting its structure, abundance, or function might compromise female fertility (Miettinen *et al.*, 2001; Yesilaltay *et al.*, 2014).

Dyslipidemia is the most common metabolic abnormality in PCOS, although the type and extent of the derangement has been variable. According to the National Cholesterol Education Program guidelines, about 70% of PCOS women have borderline or high lipid levels (Legro *et al.*, 2001). Ethnicity could represent an important determinant of the metabolic derangements in population. A considerable Danish study on a significant number of women with PCOS showed differences for the median values of total cholesterol, LDL cholesterol and triglycerides, although obtained lipid values were within the range of normality (Glintborg *et al.*, 2010).

Family history of dyslipidemia is often present in PCOS subjects. The relative risk for developing dyslipidemia is estimated to be 1.8 in PCOS family members (Diamanti-Kandarakis *et al.*, 2007). Elevated triglycerides are present in pubertal period in daughters of PCOS women, even before the onset of hyperandrogenism. In PCOS women that have a positive family history on the PCOS-related clinical abnormality, higher values of total cholesterol, LDL cholesterol, Apo B and triglycerides were found (Hu *et al.*, 2010).

1.5. Trace Element:

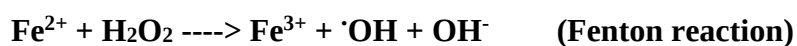
Trace elements are defined as any element that comprises less than 0.01% of the organism. Despite their small concentration, trace elements play a vital role in many different biological functions. The relationship of trace elements to different disease states has long been known (Pasha *et al.*, 2009). The World Health Organization (WHO) has classified 19 trace elements that are vital to human health including Fe, Se, Zn, and Cu

(Silvera and Rohan, 2007). Although trace elements are essential components of biological structures or essential for enzymatic function, they can be toxic at higher concentrations beyond what is necessary for their biological functions (Fraga, 2005).

The criteria for essentiality for human health is that absence or deficiency of the trace nutrient from the diet produces either functional or structural abnormalities as a result of specific biochemical changes that may be reversed by the presence of the essential nutrient (World Health Organization, 2002). Nutritional elements such as Fe, Zn, Cu, and Se are components of proteins, enzymes and hormones, which regulate many processes, including balancing redox reactions, immune response, and formation of connective tissue during development (Boffetta, 1993).

1.6.1. Iron

Iron is an element essential for life and maintaining normal organism function. In mammals, the vast majority of iron is used to synthesize hemoglobin, the oxygen-carrying protein in erythrocytes. It is also necessary for the production of myoglobin, oxidative phosphorylation within mitochondria, DNA synthesis, and making iron–sulfur-containing proteins (Pantopoulos *et al.*, 2012). While iron is critical for survival and its deficiency can lead to anemia, it is also dangerous in high amounts, as it can promote free radical damage (Walling, 1997). It plays a central role in the Fenton reaction, in which oxidation reduction (redox) reactions with hydrogen peroxide release hydroxyl and hydroperoxyl radicals.



These reactive oxygen species (ROS) promote lipid peroxidation and DNA damage (Radakovich and Olver, 2017). Because iron is necessary for life but is also toxic in excess, mammals have developed an intricate system for its regulation and availability (Drakesmith *et al.*, 2015). Transferrin is the transport protein that carries iron throughout the blood stream to reach its targets, primarily the erythroid cells of the bone marrow. Each unit of transferrin can carry 2 molecules of ferric iron (Crichton and Charloteaux-Wauters, 1987).

Iron must be bound to transferrin within the blood to prevent oxidant damage. Excess iron is stored as ferritin, primarily in the liver. (Finazzi and Arosio, 2014). Women who do not get sufficient amounts of iron may suffer anovulation (lack of ovulation) and possibly poor egg health, which can inhibit pregnancy at a rate 60% higher than those with sufficient iron stores in their blood. When the blood does not get enough iron, anemia, or an insufficient number of red blood cells, may develop (Alwais *et al.*, 2016). Since it is these red blood cells that deliver oxygen to all of the body's tissue and organs including the ovaries and uterus, this deficiency may cause the eggs stored in the ovaries to weaken over time and become unviable. Worse yet, should conception occur, anemia makes it impossible for the growing fetus cells to divide and grow properly (Sathiyarayanan *et al.*, 2014).

1.6.2. Copper

Copper is an essential trace element. Copper is found in all organs and tissues of the human body, ranging from a few ppm to several hundred ppm. It is normally bound to proteins as an essential enzymatic component. In terms of storage capacity, high concentrations of Cu are found in liver, which is the central organ for Cu homeostasis. Other organs that have high concentrations of copper are the brain, heart, stomach and intestine (Robertson , 2012).

Free Cu^+ is a potent oxidant causing the generation of ROS in cells. Thus, the tight regulation of copper homeostasis is required to maintain Cu uptake, transport, storage, and excretion activities. Cells have to vigorously bind copper to other molecules if excess copper is present. Disruption of normal copper homeostasis can result in Cu toxicity (Robertson, 2012).

Two important enzymes considered here are superoxide dismutase and cytochrome *c* oxidase (Jett and Leary, 2018). High concentrations of labile copper are problematic in terms of oxygen toxicity, as in the case of free iron, but emerging data reveal contributions of dynamic copper pools to physiological signaling (Chang, 2015).

1.6.3. Female Infertility and Trace elements

Many trace elements in biological processes, including reproduction. In the late 1990s, Xu et al. hypothesized that a disorder in the metabolism of trace elements may be one of the important factors causing unexplained infertility (Xu *et al.*, 1997). Studies of Pathak and Kapil and Ceko *et al.*, which are reviewed elsewhere, have reported that changes in the levels of the trace elements, Fe, Zn, and Ca, play important roles in female infertility, but there is still debate about the relationship between iron and unexplained infertility (Pathak and Kapil, 2004; Ceko *et al.*, 2016).

A better understanding of the role of trace elements in the underlying mechanisms in infertility, as well as more rigorous studies clarifying the effectiveness of nutritional factors, are needed to improve diagnosis and treatment (Ebisch *et al.*, 2007). Despite the paucity of studies, it is clear that there is a strong relationship between nutritional status of trace elements prior to conception and successful conception and healthy progression of pregnancy (Cetin *et al.*, 2010).

Zinc is crucial for normal insulin hormone response especially in the downstream insulin signalling. It is essential for normal insulin hormone synthesis, storage and release (Chausmer, 1998). Zinc perse has an insulin-like activity upon binding to insulin receptor (Chaves *et al.*, 2010). It has been reported that zinc supplementation successfully improved the glucose homeostasis and alleviate insulin resistance (Salgueiro *et al.*, 2001). Therefore, zinc deficiency could explain insulin resistance in women with PCOS (Guler *et al.*, 2014). Both low and high copper concentrations are linked to the development of oxidative stress through decrease antioxidant enzyme activity and development of Fenton reaction, respectively (Valko *et al.*, 2005). Published data on the trace elements, zinc and copper, in women with PCOS are scanty (Chakraborty *et al.*, 2013; Celik *et al.*, 2013; Zheng *et al.*, 2015).

1.6. Female Infertility and Genetic Variations:

Reproductive defects and genital tract developmental defects are not uncommon in women, yet little is known about the genetics behind them. During sexual development, congenital malformations of the reproductive tract may occur that affect fertility (Gaware *et al.*, 2009). Female infertility is caused by genetic, hormonal, or environmental factors. In addition, pelvic inflammatory disease, uterine fibroids, age-related factors, tubal blockage, and hostile cervical mucus can cause infertility in females (Gaware *et al.*, 2009; Olooto *et al.*, 2012). Recent findings in the genetics of female infertility are discussed below:

1.6.1. Unexplained infertility and Genetic Variations:

Unexplained infertility is likely due to multiple defects as it has been associated with numerous molecular and cellular disturbances in the endometrium. Defective uterine receptivity is thought to be a primary cause of unexplained infertility (Sharkey and Smith, 2003).

Small supernumerary marker chromosomes (sSMC) are defined as structurally abnormal chromosomes that cannot be identified or characterized unambiguously by conventional banding cytogenetics alone; they are generally equal in size or smaller than a chromosome 20 of the same metaphase spread. Small supernumerary marker chromosomes (sSMC), can lead both to fertility problems (Shah *et al.*, 2003). It was found out that after sSMC detection in connection with unexplained infertility in ~60% of cases the origin of the sSMC can be characterized by application of the centromere-specific probes for chromosomes 14 and 15. (Manvelyan *et al.*, 2008).

Some patients with unexplained infertility, the level of phosphorylated (Signal transducer and activator of transcription) STAT3 appears to be abnormally low in their endometrium (Dimitriadis *et al.*, 2007). Phosphorylation of murine STAT3 is induced by embryo implantation (Teng *et al.*, 2004) and the inhibition of STAT3 activity is associated with implantation failure due to the absence of stromal cell decidualisation (Nakamura *et al.*, 2006).

The (Methylene tetrahydrofolate reductase) MTHFR genetic variants are also associated to female unexplained infertility (Altmäe *et al.*, 2010). MTHFR has an important role in folate metabolism and DNA methylation in a cell. The MTHFR enzyme participates in the conversion of homocysteine to methionine, a precursor for the methylation of DNA, lipids, and proteins (Gomes Andrea, 2015) Polymorphisms in folate-metabolizing pathways of genes may account for the unexplained infertility seen in these women, as it disrupts homocysteine levels and subsequently alters homeostatic status. Impaired folate metabolism disturbs endometrial maturation and results in poor oocyte quality (Altmae *et al.*, 2010). A polymorphic variation of the (The transmembrane mucin glycoprotein) *MUC1* gene has been associated

with female infertility due to suspected failure of embryo implantation, based on the significant greater size of the lower allele observed in women with unexplained infertility (Horne *et al.*, 2001).

1.6.2. PCOS and Genetic Variations:

The interaction between multiple genetic and environmental factors is probably necessary for the development of PCOS. Several lines of research suggest that there is a genetic component to the pathophysiology of the syndrome (Baillargeon and Carpentier, 2007). A relation between PCOS with the X chromosome aneuploidies and polyploidies in addition to other cytogenetic abnormalities has been confirmed. (Hickey *et al.*, 2002).

The concept is that at least some cases of PCOS may be due to X chromosomal factors causing an abnormal follicular apparatus. In addition, large deletion of the long arm of chromosome 11 was seen in some of the PCOS cases. However, there is no large cytogenetic study to identify karyotype abnormalities (Sheikhha *et al.*, 2007).

The candidate-gene approach to genetic association studies of PCOS is based on the biological plausibility of potential genes. Primary candidates include: (i) genes involved in the biosynthesis and activity of androgens; (ii) genes affecting insulin resistance and (iii) genes correlated with inflammatory cytokines. Because this topic has been well-documented previously (Zhao and Chen, 2013).

Hyperandrogenism is a fundamental characteristic and clinical manifestation of PCOS. For this reason, genes involved in steroidogenesis (e.g. CYP11A, CYP17A, CYP21, EPHX1, and HSD17B6) have been regarded as essential candidates. Sex hormones and hormone receptors play a fundamental role in folliculogenesis. Therefore, FSH, FSHR, LHCGR, AR and SHBG are regarded as compelling candidate genes for PCOS (Valkenburg *et al.*, 2009; Du *et al.*, 2010).

1.8. Aim of The Study:

This study was designed to achieve several aims relevant to unexplained and PCOS infertility Iraqi women in (Thi-Qar government). Including general aims:

First:-Investigation the pathogenic role of several biochemical parameters in the UI women:-

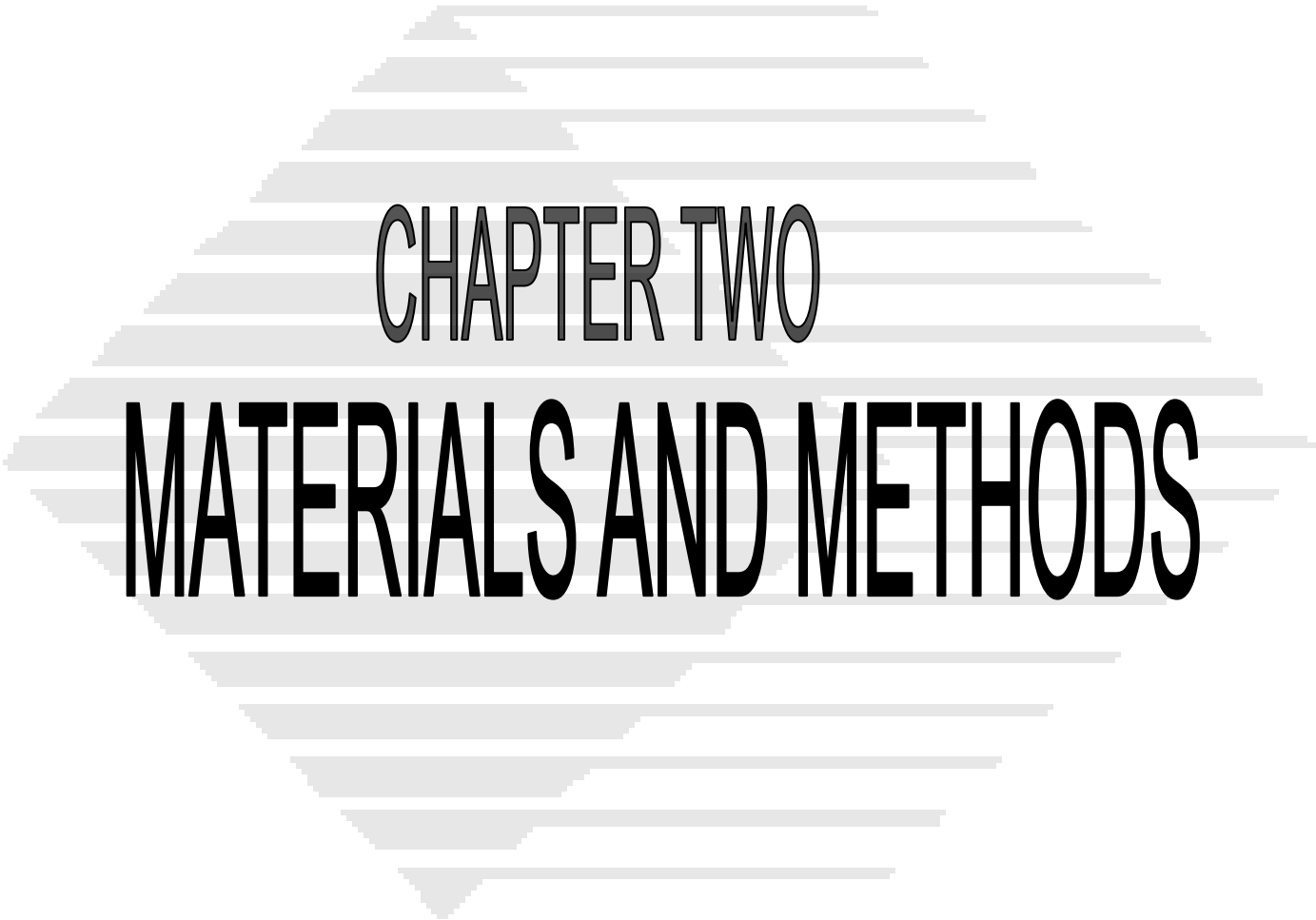
1. Oxidative stress that represented by lipid peroxidation marker (MDA).

And antioxidants that represented by GSH, SOD and Cp

2. Some trace elements (iron and copper).

Second:- Detection the pathogenic role of GSR gene, mutation and polymorphism in UI women.

Third:- Investigation of the possible relationships between each one of the biochemical parameters and polymorphism GSR gene.



CHAPTER TWO

MATERIALS AND METHODS

2.1.Chemicals:

The chemicals that were used in this study with their companies are showed in table (2-1).

Table (2-1): Chemicals and Their Suppliers

<i>NO.</i>	<i>Chemicals</i>	<i>Supplied Company</i>
1	6X Loading dye	Intron / Korea
2	Agarose	Conda / USA
3	Bradford Protein Assay Kit	Tonk Biosciences USA
4	Cholesterol Kit	Biolabo,France
5	Copper Kit	Spectrum, Germany
6	DTNB	BDH (England)
7	FSH Kit cobas e 411	Roche Diagnostics, Germany
8	G- spin DNA extraction kit	intron biotechnology/Korea
9	glacial acetic acid	BDH, England
10	HDL Kit	Biolabo,France
11	Iron kit	Randox,UK
12	Ladder 100bp	Kapa /USA
13	LH Kit cobas e 411	Roche Diagnostics, Germany
14	Na ₂ HPO ₄	FlukaGarnatie (Switzerland)
15	NaH ₂ PO ₄	FlukaGarnatie (Switzerland)
16	Para pheneline diamine(PPD)	BDH, England

17	Pre mix pcr	Intron / Korea
18	Primer	Integrated DNA technologies /USA
19	Prolactin II Kit of cobas e 411	Roche Diagnostics, Germany
20	Red safe staining souluion	Intron / Korea
21	Sodium acetate trihydrate	BDH, England
22	Sodium azide	Reidledehain, Germany
23	Superoxide Dismutase Assay Kit	Cohesion Biosciences USA
24	TBE buffer 10 X	Conda / USA
25	Thio bar bituric acid(TBA)	BDH, England
26	Tri chloro acetic acid(TCA)	BDH, England
27	Triglyceride Kit	Biolabo,France
28	TSH Kit cobas e 411	Roche Diagnostics, Germany

2.2. Collection of Blood Sample:

Blood samples were obtained from fasting infertile women and control group by venepuncture, using a 10 ml disposable syringe. The blood sample was divided into two aliquots, which were 8 and 2 ml. The first aliquot was dispensed in a plain tube, and left for 15 minutes at room temperature to clot. Then, it was centrifuged at 3000 rpm for 10 minutes to collect serum and kept in the freezer (-20°C) until use unless used immediately to analyze biochemical parameters. The second aliquot was dispensed in a sterilized tube with EDTA to prevent coagulation and this used to detection of glutathione-disulfide reductase GSR.

2.3. Design of Study:

This study has been conducted at AL-Hussein Teaching Hospital in Thi-Qar, Biochemistry Laboratory at College of Science, during the period between 10/8/2016 to 25/5/2017. Informed consent was obtained verbally from all participants. A total of (136) women with infertility of the ages (20 - 35) years were selected. The women were already diagnosed as infertile women by the consultant medical staff; According to clinical examination and symptoms (68) women suffer from unexplained infertility (UI) whereas (68) women suffered from polycystic ovarian syndrome (PCOS). To get the normal values of studied parameters, the study included (34) healthy ages matched fertile women with a history of at least one child birth were also enrolled. All the mentioned subjects (170) females, which divided into two general groups:

The details of the numbers and age of the two groups are illustrated in table (2.2).

Table (2.2): Details of numbers and age of the studied groups

<i>Groups</i>	<i>No.</i>	<i>Rang of Age (years)</i>
Patients (infertile women)	136	20-35
Control (fertile women)	34	20-35
Total	170	20- 35

Also the women in this study were divided into subgroups according to type of infertility of infertile women showed as followings:

(1°UI): included (34) primary unexplained infertile women who have no previous pregnancies.

(2° UI): included (34) secondary unexplained infertile women who have previous pregnancies.

(1°PCOS): included (34) primary infertile women (PCOS) who have no previous pregnancies.

(2° PCOS): included (34) secondary infertile women (PCOS) who have previous pregnancies.

It is notable that the clinical chart of this study describes experimental parameters and genes in the following table (2-2).

Table (2-3): The Clinical Chart of the Study

Patient Profile
Name :
Age:
Type of infertility:
Primary:
Secondary:
Resident:
BMI (kg/h ²):
Drug:
Others:
Biochemical Test
Lipid profile: TG, TC, HDL, LDL, VLDL,
Lipid peroxidation: MDA
Anti-oxidant: CP, GSH
Trace element: Fe, Cu
Hormones: PRL, TSH, FSH, LH
Detection of glutathione disulfide reductase <i>GSR</i> gene

2.2. Instrument

The instruments which were used in this study and their suppliers shown in Table (2-4).

Table (2-4): The Instruments and their manufacturers

NO.	Instruments	Manufacturers
1	AURA TM PCR Cabinet	Italy
2	Balance	Kernpfb, Germany
3	Beckman Coulter AD 340	Australia
4	Bio TDB-100, Dry block thermostatbuilt	Bio San, Germany
5	Biopette Variable Volume 2-20 ul	Germany
6	Centrifuge	Universal 16A , Germany
7	Combi-spin	Biosan, Lativa
8	Document system	Labnet, USA
9	Electrophoreses	CBS, Scientific, USA
10	Incubator	Jard, Japan
11	Microspin	Biosan, Lativa

12	Microspin 12, High-speed Mini-centrifuge	Bio San, Germany
13	Microwave	Gosonic, China
14	Mini-Power Supply 300V, 2200V	Chain
15	Multi Gene OptiMax Gradient Thermal Cycler	Labnet, USA
16	Sensitive balance	MO.12307078, Japan
17	Shacking water bath	SW23,Germany
18	Nanodrop 1000	(Quawell / Hong kong)
19	The immunoassay Analyzer cobas e 411 2 nd generation platform of ECL technology	Roche Diagnostics GmbH D-68298 Mannheim, Germany
20	UV.transmission	Vilber lourmat, Farance
21	UV/VIS spectrophotometer	UV-7804C, Japan
22	UV/VIS spectrophotometer	T 60, PG Instruments Ltd, Germany
23	V-1 plus, Personal Vortex for tubes	Digsystem, Germany
24	Vortex	VELP Scientific, EVROPE

2.5 Methods:

2.5.1 Biochemical Parameters:

Several considerable methods were used to measure the studied parameters. It is notable that all measurements were duplicated for each sample.

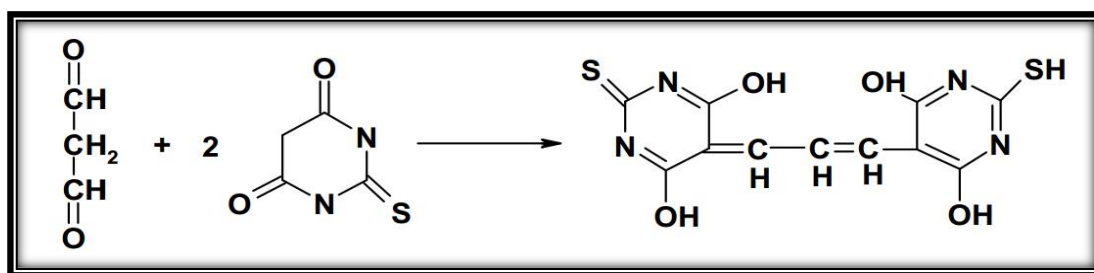
2.5.1.1 Determination of Serum Hormones:

The Hormones (PRL, TSH, FSH, and LH) are a full automated quantitative test for use with Autoanalyzer cobas e411 instruments were used for the determination of (PRL TSH, FSH, and LH) in human serum or plasma using Elecsys and cobas e immunoassay analyzers.

2. 5.1.2. Determination of Serum Malondialdehyde (MDA)

Principle:

Lipid peroxidation was determined by using the thiobarbituric acid method. In this method, malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids were identified as the product of LPO that react with thiobarbituric acid (TBA), in coexisting trichloro acetic acid (TCA), to give a red chromophore absorbing at 532 nm (Fong *et al.*, 1973). MDA concentrations were calculated, using the molar extinction coefficient of MDA (ϵ_{MDA}) equal to $1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Malondialdehyde (MDA) formed from breakdown of polyunsaturated fatty acid, serves as a convenient index of peroxidation reaction.



Procedure:

The level of serum MDA was determined spectrophotometrically with a thiobarbituric acid (TBA) solution, to 150 μl serum sample were added to 1ml of 17.5% trichloroacetic acid (TCA) and 1ml of 0.66% TBA, mixed well by vortex, incubated in boiling water for 15 minutes, and then allowed to cool. One ml of 70% TCA was added and the mixture allowed to stand at room

temperature for 20 minutes, centrifuged at 2000 rpm for 15 minutes, the supernatant was taken out for scanning spectrophotometrically at (532nm) (Muslih *et al.*, 2002). The concentration of MDA calculated as follow:

$$MDA \left(\frac{\mu mol}{L} \right) = \frac{A}{L \times \epsilon} \times D \times 10^6$$

A: absorbance

L: light path (1cm)

ε: Extinction coefficient $1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1} = 6.7$

D: Dilution factor = 1 mL Vol. Used in ref./0.15

2. 5.1.3. Determination of Antioxidant Parameters:

2. 5.1.3.1. Determination of Serum Reduced Glutathion (GSH):

Concentration:

Principle:

The plasma thiol concentration was measured according to Ellman's method (Ellman, 1959). This method depends on the reaction of 5,5-Dithio bis (2-nitrobenzoic acid) (DTNB) with aliphatic thiol compounds at pH 8.0 to produce one mole of p-nitrothiol phenol anion per mole thiol. Since this anion highly colored ($\epsilon=13600$ at 412 nm), it can be used to measure the thiol concentration.

Reagents:

1- Phosphate buffer solution with pH 7 and the concentration of 0.2 molar consists of:

- a)** Solution A: the solution of 0.2 molar acidic Sodium phosphate (NaH_2PO_4) is prepared by dissolving 2.4 mg of (NaH_2PO_4) into a volume of water and completed to 100 ml.

- b)** Solution B: the solution of 0.2 molar basic Sodium phosphate (Na_2HPO_4) is prepared by dissolving 2.83 mg of (Na_2HPO_4) into a volume of water and completed to 100 ml.

In order to prepare the buffer solution, 19.5 ml of the solution A was mixed with 30.5 ml of the solution B and completed to 100 ml of the solution with pH 7.

- 2-** Phosphate buffer solution with pH 8 and the concentration of 0.2 molar :

This buffer solution is prepared by mixing 2.62 ml of 0.2M (NaH_2PO_4) with 47.38 ml of 0.2 M (Na_2HPO_4) and completed to 100 ml of pH 8 solution.

- 3-** DTNB solution (5, 5-dithio-bis (2-Nitrobenzoic acid)) which is prepared by dissolving 39.6 mg of DTNB into 10 ml of the phosphate buffer solution pH 7.

Procedure:

- 1- 0.02 ml of the plasma was added to 9 ml of water.
- 2- 1 ml of phosphate buffer solution pH 8 was added to the solution.
- 3- The previous solution was mixed 3 ml of with 0.02 ml of DTNB solution.
- 4- The result solution was kept for 60 seconds at the room temperature.
- 5- The equivalent solution (Blank) was prepared as the steps 1 and 2.
- 6- The absorbance of the result solution was measured by using optical spectrum at the wavelength of 412 nm.

The Calculation:

The total concentration of the Glutathione can be calculated as:

$$\text{GSH} \left(\frac{\mu\text{mol}}{\text{L}} \right) = (\text{T} - \text{B}) \times \frac{\text{D}}{\epsilon} \times 1000$$

Where: **T**: the absorbance of the solution.

B: the absorbance of the equivalent solution (Blank), **D**: the dilute factor.

ε: Molar extension coefficient equal to 13600 mol⁻¹L cm⁻¹, $\frac{\text{D}}{\epsilon} = 36.8$

2.5.1.3.2 Determination of Serum Superoxide Dismutase (SOD) concentration:

1- Determination of serum protein by colorimetric protein assay:

Principle:

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford 1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (A_{max} = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (A_{max} = 595 nm) It is this blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer or microplate reader.

Reagents Composition:

Component	Volume	Storage
G250 dye reagent	100 mL × 2	4 °C
BSA standard (5 mg/ml)	1 mL	-20 °C
Handbook	1	

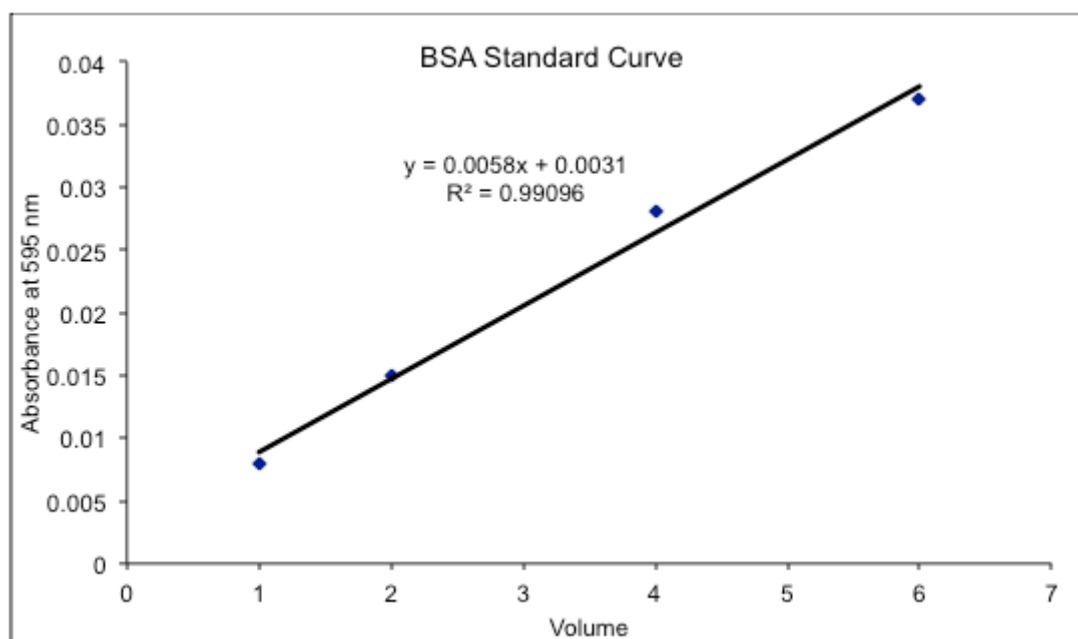
Procedure:

1. (5mg/ml) BSA standard was diluted to 0.5 mg/ml. Example: add 10µL BSA standards to 90µL 0.9% NaCl or PBS dilution standard.
2. Pipetted 0, 1, 2, 4, 8, 12, 16 and 20 µl of BSA (0.5 mg/ml) into assigned wells of a 96-well plate.
3. Pipetted X µl of unknown sample into individual wells of a 96-well plate.
4. Double distilled water was added to all wells to make the final volume to 20µl
5. (200) µl of Coomassie Blue G250 dye reagent was added into all wells containing standard and sample, and incubated at room temperature for 3-5 minutes. The operation was according to the flowing table.

Well	1	2	3	4	5	6	7	8	9
Concentration of BSA (µg/ml)	0	25	50	100	200	300	400	500	Unknown sample
BSA standard (0.5mg/ml)/unknown sample (µl)	0	1	2	4	8	12	16	20	X
Double distilled water (µl)	20	19	18	16	12	8	4	0	20-X
G250 dye reagent (µl)	200	200	200	200	200	200	200	200	200

6. Absorbances were read at 595 nm wavelength.

7. The concentrations of unknown samples were calculated according to standard curve and X.



Bovine serum albumin (BSA) standard curve

2- Determination of Serum (SOD):

Principle:

Superoxide Dismutase Microplate Assay Kit, ions generated from the conversion of xanthine to uric acid, and hydrogen peroxide by xanthine oxidase (XOD), converts Nitro Blue Tetrazolium NBT to NBT-diformazan. NBT-diformazan absorbs light at 560 nm. SODs reduce superoxide ion concentrations and thereby lower the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT-diformazan is a measure of SOD activity present in experimental samples (Robak and Gryglewski,1988).

Reagents Composition:

Component	Volume	Storage
96-Well Microplate	1 plate	
Reaction Buffer	5 mL x 1	4 °C
Substrate	Powder x 1	4 °C
Enzyme	100 µl x 1	4 °C
Dye Reagent	5 mL x 1	4 °C
Standard	Powder x 1	-20 °C
Plate Adhesive Strips	3 Strips	
Technical Manual	1 Manual	

Procedure:

The following reagents were added into the microplate:

Reagent	Sample	Standard	Blank
Reaction Buffer	30 µl	30 µl	30 µl
Substrate	100 µl	100 µl	100 µl
Enzyme	1 µl	1 µl	1 µl
Sample	19 µl	--	--
Standard	--	19 µl	--
Distilled water	--	--	19 µl
Dye Reagent	50 µl	50 µl	50 µl

Mixed, stand at room temperature for 30 minutes, recorded absorbance measured at 560 nm.

Calculation:

According to the protein concentration of the sample:

$$\text{SOD (U/mg)} = (\text{C Standard} \times \text{V Standard}) \times (\text{OD Blank} - \text{OD Sample}) / (\text{OD Blank} - \text{OD Standard}) / (\text{V Sample} \times \text{C Protein})$$

$$= 30 \times (\text{OD Blank} - \text{OD Sample}) / (\text{OD Blank} - \text{OD Standard}) / \text{C Protein}$$

C Protein: the protein concentration, mg/ml

C Standard: the standard activity, 30 U/ml

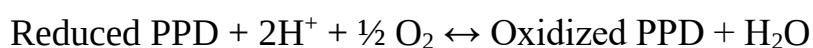
V Standard: the volume of standard, 0.019 ml

V Sample: the volume of sample, 0.019 ml

2.5.1.3.3. Determination of Serum Ceruloplasmin Concentration

Principle:

Serum Cp concentration was measured by the method of (Menden *et al.*, 1997). It is based on the ceruloplasmin-catalyzed oxidation of colorless para-phenylenediamine (PPD) to blue-violet oxidized form. The reaction is followed photometrically and the blank value is determined after inhibition of the enzyme with sodium azide at (0°C).



(Colorless)

(Blue)

A mixture of serum, substrate and acetate buffer at pH = 6.0 was incubated at 37°C for 15 min. The reaction is stopped by the addition of sodium azide, and the absorbance of the purple color formed (Oxidized PPD) in the diluted test mixture is read at 525 nm against blank solution. The corrected absorbance is directly related to the concentration of Cp (Ravin, 1961).

Reagents:

- **Substrate solution:**

50 mg of PPD was dissolved in 5 mL (4 mL of distilled water (D.W) and 1 mL of glacial acetic acid). In another container 8.15gm sodium acetate trihydrate was dissolved in 30 mL of D.W then added to first solution, mixed

and completed the volume to 50 mL with distill water This solution is stable for three hours and is kept refrigerated in a dark bottle.

- **Inactivating solution:**

100 mg of sodium azide was dissolved in (500 mL) of D.W then was kept cold in a refrigerator prior to use, this solution must be prepared every week.

Procedure:

One mL of substrate was pipetted into glass test tubes, incubated at 37°C for 1 min then (0.1 mL) of serum was added, incubated at 37°C for 15 min, then tubes were removed and placed in iced-bath for 30 sec. five mL of cold inactivating solution was added, mixed under 25°C in water bath. Blank was prepared by combining the substrate, inactivating solution, and 0.1 mL of distil water, then the mixture was incubated as above. The absorbance of test and blank was read in spectrophotometer at 525nm.

The concentration of Cp was calculated as following:

$$Cp \left(\frac{mg}{L} \right) = \frac{A \text{ test} - A \text{ blank}}{\epsilon_{Cp}} \times 10$$

: Absorbance of test **A test**

: Absorbance of blank **A blank**

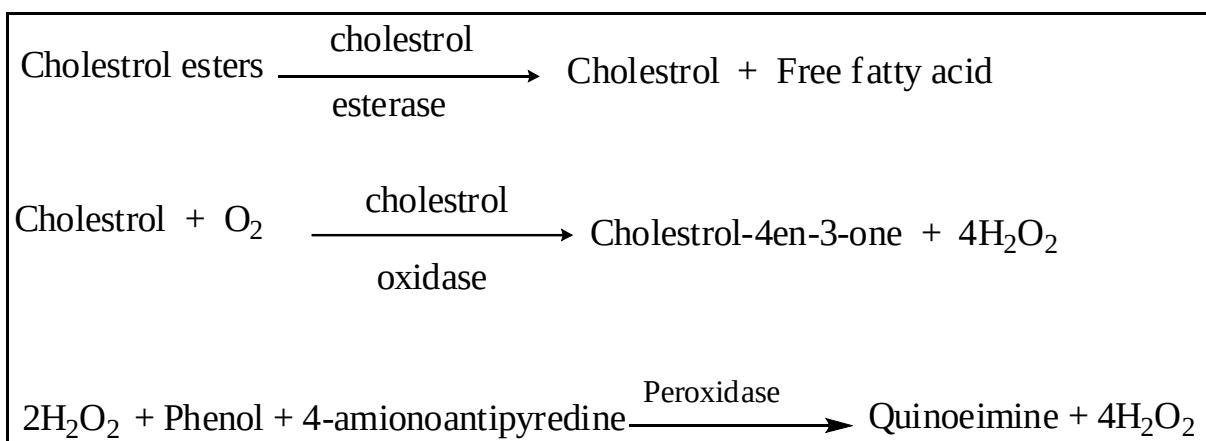
ϵ_{Cp} : The extinction coefficient of Cp equal to (0.68).

2. 5.1.4. Determination of Serum Lipid profile:

2. 5.1. 4.1. Determination of Serum Total Cholesterol (TC)

Principle:

Enzymatic method described by(Allan and Dawson 1979)which the reaction scheme is as follows :-



Reagents composition:

Reagent	Composition	
Reagent 1 Buffer	Phosphate buffer	100 mmol/L
	Chloro-4-phenol	5 mmol/L
	Sodium cholate	2.3 mmol/L
	Triton x 100	1.5 mmol/L
	Preservative	
Reagent 2 enzymes	Cholesterol oxidase	≥100 IU/L
	Cholesterol esterase	≥ 170 IU/L
	Peroxydase	≥ 1200 IU/L
	4-aminoantipyrine	0.25 IU/L
	PEG 6000	167 μmol/L
Cholesterol Cal.	Cholesterol aqueous primary standard 200 mg/dL	

Manual Procedure:

1. Reagent (1) and reagent (2) were mixed to form working solution.
2. Reagent and sample were left stand at room temperature.

	Volume	Storage	Assay
Reagent	1 mL	1mL	1mL
D.W	10 µL	-	-
Standard	-	10 µL	-
Sample	-	-	10µL

Mixed. Left stand for 5 minutes at 37°C or 10 minutes at room temperature.
Absorbance was recorded at 500 nm against reagent blank.

Calculation:

The result was calculated as the following:

$$\text{Cholesterol (mg/dL)} = \frac{A (\text{Sample})}{A (\text{Standard})} \times n$$

n: standard concentration = 200 (mg/dl)

2.5.4.2. Determination of Serum LDL Concentration

According to (Friedwald *et al.*, 1972), LDL concentrations were measured as follows:

$$\text{LDL (mg/dl)} = \text{Total cholesterol} - (\text{HDL} + \text{VLDL})$$

2.5.1.4.3. Determination of Serum HDL Concentration:

Principle:

According to (Tietz, 1999), Low density lipoproteins (LD), very low density lipoproteins (VLDL) and chylomicrons from the sample are

precipitated by phosphotungstic acid and magnesium chloride. HDL obtained in supernatant after centrifugation is then measured with total cholesterol reagent.

Reagents composition:

Reagent	Composition	
Reagent	Phosphotungstic acid	13.9 mmol/L
Precipitant	Magnesium chloride	570 mmol/L
Reagent 2	Cholesterol	2.58 mmol/L
Standard		

Manual procedure:

Sample preparation (without treating standard):

Pipetted in centrifuge	Micromethod
Sample	500 µL
Precipitant	50 µL

Mixed vigorously, Waited for 10 minutes at room temperature, centrifuged for 15 minutes at 3500 – 4000 rpm, aspirate the supernatant. Reagent and supernatant was left stand at room temperature.

Component	Volume	Storage	
Reagent2 (Cholesterol assay)	1 mL	1 mL	1 mL
D.W	25 µL	-	-
Standard	-	25 µL	-
Supernatant (HDL)	-	-	25 µL

Mixed. Left stand for 5 minutes at 37°C or 10 minutes at room temperature. Absorbance was recorded at 500 nm against reagent blank.

Calculation:

The result was calculated as the following:

$$\text{HDL (mg/dL)} = \frac{A (\text{Assay})}{A (\text{Standard})} \times n \times 1.1$$

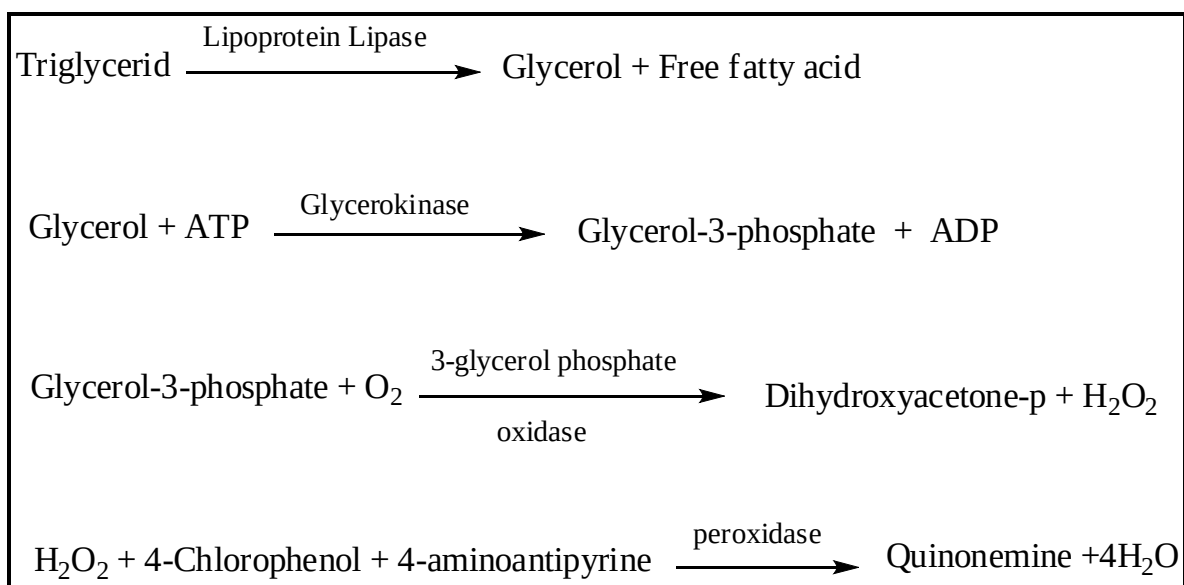
n: standard = 100 (mg/dL)

2.5.1.4.4. Determination of Serum Triglyceride (TG):

The used reagents were supplied by Biolabo (France), and Serum TG was measured according to (Fossati and Prencipe, 1982).

Principle:

Fossati and Prencipe method associated with Trinder (Fossati and Prencipe, 1982), according to the following reactions, Reaction scheme is as following:



Reagents composition:

Reagent	Composition	
Reagent1 Buffer	Pipes	10 mmol/L
	Magnesium chloride	9.8 mmol/L
	Chloro-4-phrnl	3.5 mmol/L
Reagent2 enzymes	Lipase	≥1000 IU/L
	Peroxidase	≥ 1700 IU/L
	Glycerol-3-phosphate oxidase	≥ 3000 IU/L
	Glycerol kinase	≥ 660 IU/L
	4-aminoantipyrine	0.5 mmol/L
	ATP	1.3 mmol/L
Reagent 3 standard	Glycerol	2.28 mmol/L (200 mg/dl)

Manual procedure:

1. Reagent (1) and reagent (2) were mixed to form working solution.
2. Reagent and sample were left stand at room temperature.

	Blank	Standard	Assay
Reagent	1 mL	1 mL	1 mL
D.W	10 µL	-	-
Standard	-	10 µL	-
Sample	-	-	10 µL

Mixed. Left stand for 5 minutes at 37°C or 10 minutes at room temperature.
Absorbance was recorded at 500 nm against reagent blank.

Calculation:

The result was calculated as the following:

$$\text{TG (mg/dL)} = \frac{\text{A (Sample)}}{\text{A (Standard)}} \times n$$

n: standard concentration = 200 (mg/dl)

2.5.1.4.5 Determination of Serum VLDL Concentration:

According to (Friedwald *et al.*, 1972), VLDL concentrations were measured as following:

$$\text{VLDL (mg/dl)} = \text{serum TG} / 5$$

2.5.1.5. Determination of Serum Trace Element Concentration:

2.5.1.5.1 Determination of Serum Iron Concentration:

Principle:

Ferric iron was dissociated from its carrier protein, transferrin, in an acid medium and simultaneously reduced to the ferrous form. The ferrous iron is then complexes with the chromogen, a sensitive iron indicator, to produce a blue chromophore which absorbs maximally at 595 nm (Ceriotti *et al.*,1980).

Reagents Composition:

Reagent	Contents	Concentration of solutions
R1	Ferene	22.2mmol/L
Chromogen		
R2	Ascorbic acid	1.3 mol/l
Reductant		
R3	Acetate buffer	0.087 mol/l,pH 4.65
Buffer	Dimethyl sulphoxide	
	Surfactant	

Procedure:

	blank	Sample	Standard
Buffer	1.00 mL	1.00 mL	1.00 mL
Reductant	0.05 mL	0.05 ml	0.05 ml
Iron – free water	0.25 ml	-	-
Standard	-	-	0.25 ml
Sample	-	0.25 ml	-

Mixed, read of the sample and of the standard against the reagent blank.

Chromogen	0.05 ml	0.05 ml	0.05 ml
------------------	---------	---------	---------

Mixed, incubated for at least 15 min at 20 -25°C. Final absorbance was read against reagent blank.

Calculation:

The results calculated as following:

$$\text{Iron conc. (}\mu\text{g/dL)} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{Conc. of standard}$$

ΔA = Final Absorbance -Initial Absorbance

2.5.1.5.2. Determination of Serum Copper Concentration:

Principle:

Copper forms with 4-(3,5-dibromo-2-pyridylazo)-N-ethyl-sulfopropylaniline a chelate complex . The increase of absorbance of this complex can be measured and is proportional to the concentration of total copper in the sample (Abe and Yamashitha, 1989).

Reagents Composition:

Reagents	Contents	Concentration
R (Monoreagent)	Acetate buffer PH 5.0	0.2 mol/L
	4-(3,5-dibromo-2-pyridylazo)-N-ethyl-sulfopropylaniline	0.02 mmol/L
Standard (ST)		100 µg/dL

Procedure:

	Blank	Standard	Sample
Reagent	1.0ml	1.0 ml	1.0 ml
Standard	-	50 µl	-
Sample	-	-	50 µl
D.W	50 µl	-	-

Mixed and incubated for 5 minutes at 37 °C. Measured the absorbance of the sample A_s and of the standard A_{st} against the reagent blank A_{RBL} .

Calculation:

$$\text{Copper conc. } (\mu\text{g/dL}) = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{conc. of standard}$$

$$\Delta A_s = A_s - A_{\text{RBL}}$$

$$\Delta A_{\text{std}} = A_{\text{std}} - A_{\text{RBL}}$$

2.5.2 Detection of GSR Gene:

2.5.2.1 DNA Extraction:

G- spin DNA extraction kit , Intron Biotechnology/Korea , Cat.No. 17045

Kit Contents:

Label	Contents 50 Columns
Buffer CL	25 mL
Buffer BL	25 mL
Buffer WA	40 mL
Buffer WB	10 mL
Buffer CE	20 mL
Spin Column / Collection Tube	50 ea
RNase A (Lyophilized powder)	3 mg x 1 vial
Proteinase K (Lyophilized powder)	22 g x 1 vial

Protocol (according to intron Biotechnology Company):

Isolation of DNA from whole blood:

- 1- 200µl of culture of blood was pipeted into a 1.5 ml microcentrifuge tube.
- 2- 20µl of Proteinase K and 5µl of Ribonuclease RNase A solution were added into sample tube and gently mixed.
- 3- 200µl of Buffer BL was Added into upper sample tube and mixed thoroughly. And any vigorous vortexing was avoided because doing so

may induce genomic DNA breakage. The lysate was incubated at 56°C for 10 min. For complete lysis, mixed 3 or 4 times during incubation by inverting tube. Briefly the 1.5 ml tube was centrifuged to remove drops from the inside of the lid.

- 4- 200µl of absolute ethanol was added into the lysate, and mixed well by gentle inverting for 5 - 6 times or by pipetting. DO NOT vortex. After mixing, briefly centrifuged the 1.5 ml tube to removed drops from inside of the lid.
- 5- The mixture from step 4 to the spin column was carefully applied (in a 2 ml collection tube) without wetting the rim, the cap was closed, and centrifuged at 13,000 rpm for 1 min. The filtrate and the spin column placed in a 2 ml collection tube (reuse) were discarded.
- 6- 700µl of Buffer WA was added to the Spin Column without wetting the rim, and centrifuged for 1 min at 13,000 rpm. The flow-through and reuse the Collection Tube were discarded.
- 7- 700µl of Buffer WB was added to the Spin Column without wetting the rim, and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and placed the Column into a 2.0 ml Collection Tube (reuse), then again centrifuged for additionally 1 min to dry the membrane. The flow-through and Collection Tube were discarded altogether. Note: Ensure that 40 (160) ml of absolute ethanol was added to Buffer IWB.
- 8- The spin column was placed into a new 1.5 ml tube (not supplied), and 30 - 100µl of Buffer CE directly onto the membrane. Incubated for 1 min at room temperature and then centrifuged for 1 min at 13,000 rpm to elute.

2.5.2.2. Agarose Gel Electrophoresis of DNA:

Electrophoresis has been done to determine DNA bands after the process of extraction or to detect the result of the interaction of PCR during the

presence of the standard DNA to distinguish the band size of the outcome of the interaction of PCR on the agarose gel.

2.5.2.2.1. Prepare of the Agarose Gel:

According to (Sambrook *et al.*, 1990), the agarose gel was made in 0.8% condensation by melting 8g of agarose in 100 ml of previously made TBE buffer. Agarose was heated to boil then left to cool down at (45-50°C). The gel was poured in the pour plate in which the plate of agarose support was prepared after fixing the comb to make holes that would hold the samples. The gel was poured gently not to make air bubbles and left 30 minutes to cool down. The comb was removed gently of the solid agarose. The plate was fixed to its stand in the electrophoresis horizontal unit represented by the tank used in the electrophoresis. The tank has been filled with TBE buffer in which it covers the gel surface.

2.5.2.2.2. Preparation of sample:

3 µl of the processor loading buffer (Intron / Korea) was mixed with 5 µl of the supposed DNA to be electrophoresis (loading dye), after the mixing process, the process of loading is now to the holes of the gel. An Electric current of 5v/cm² was exposed for 1-2 h till the tincture was reached to the other side of the gel. The gel was tested by a UV source with 336 nm after put the gel in pool contain on 30µl Red safe Nucleic acid staining solution and 500 ml from distilled water.

2.5.2.3. Measurement of DNA Concentrationq and purity:

Extracted DNA concentration and purity were measured by the nanodrop (Quawell/Hong Kong), 2µl of DNA was loaded to the lens of the nanodrop and measured on 260/280 nm wave length, the result appeared on the laptop screen that attached to the nanodrop. The nanodrop lens was cleaned by distilled water (D.W) and cotton swap after each sample; the

other samples were measured consequently. Blank solution for nanodrop was elution buffer.

2.5.3. The Primers Used in The Interaction:

The primers were lyophilized, they was dissolved in the free ddH₂O to give a final concentration of 100 pmol/μl as stock solution and was kept a stock at -20 to prepare 10 pmol/μl concentration as work primer suspended, 10 μl of the stock solution in 90 μl of the free ddH₂O water to reach a final volume 100 μl, was investigated by IDT (Integrated DNA Technologies company, Canada).

The specific primer of GSR (Integrated DNA technologies /USA):

Primer	Sequence	T _m (°C)	GC (%)	Product size
Forward	5'-GGAAGTCAACGGGAAGAAGTT-CACTG- 3'	59.4	50.0	250 base pair
Reverse	5'CAATGTAACCGGCACCCACAATAAC- 3'	58.5	48.0	

2.5.4. Maxime PCR PreMix kit (i-Taq) 20μlrxn:

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution. *Maxime* PCR Pre Mix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq DNA polymerase, dNTP mixture, reaction buffer, and so on-in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every component for PCR; PCR is just adding a template DNA, primer set, and D.W. The second reason is that it has Gel loading buffer to do electrophoresis, It is suitable for various sample's experience by fast and simple using method.

The Components of the Maxime PCR PreMix kit (i-Taq), Intron / Korea:

Material	Volume
i-Taq DNA Polymerase	5U/μl
dNTPs	2.5mM
Reaction buffer (10X)	1X
Gel loading buffer	1X

2.5.5. Diagnosis of Gene:

Mixture of the specific interaction for diagnosis GSR, (Igor et al., 2013):

Components	Concentration
Taq PCR PreMix	5μl
Forward primer	10 picomols/μl
Reverse primer	10 picomols/μl
DNA	1.5μl
Distill water	16.5 μl
Final volume	25μl

The optimum condition of detection GSR:

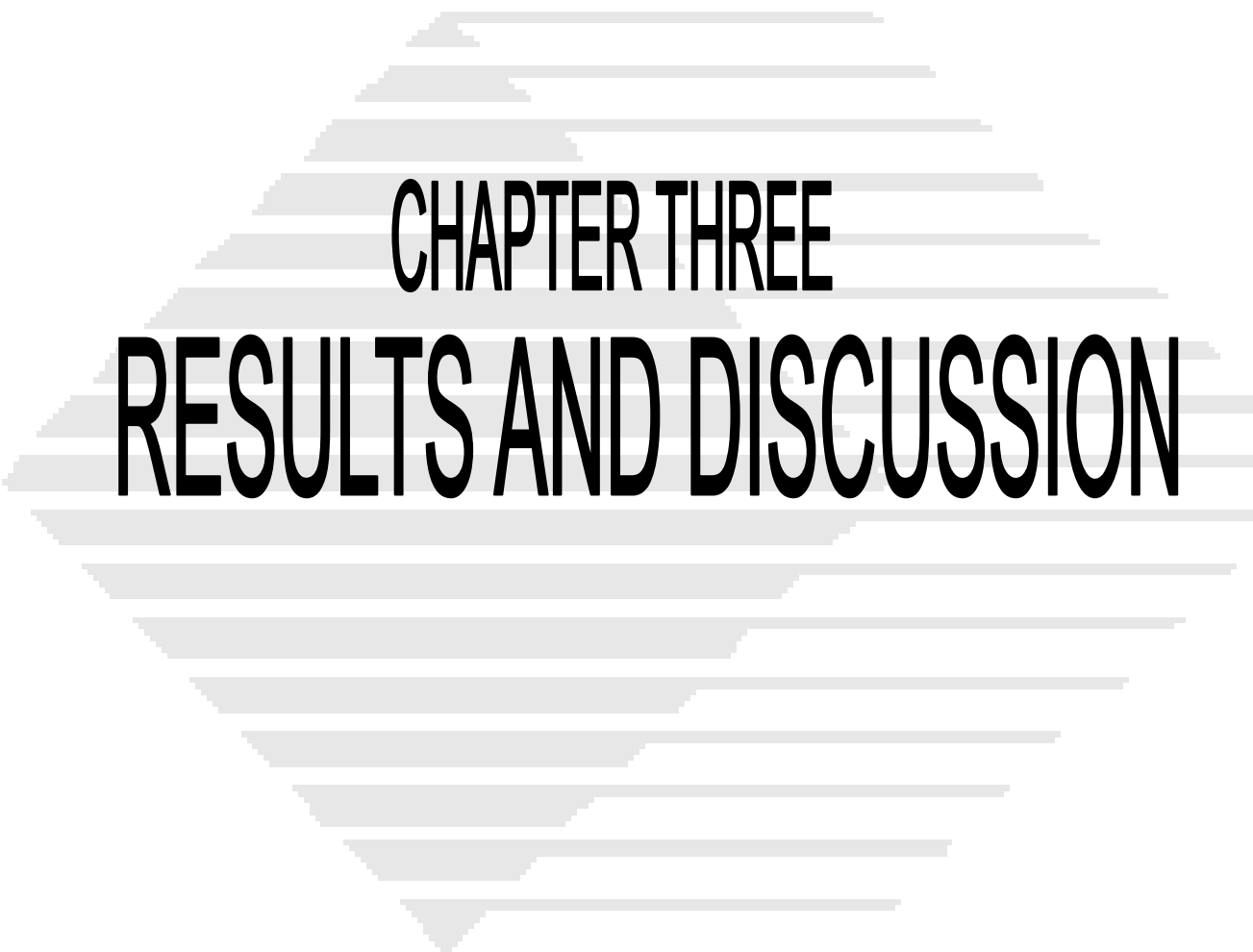
No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	3 min.	40 cycle
2-	Denaturation -2	95°C	45sec	
3-	Annealing	58°C	45sec	
4-	Extension-1	72°C	50sec	
5-	Extension -2	72°C	10 min.	

2.5.6. Sequencing and Sequence Alignment:

The PCR products were separated on a 2% agarose gel electrophoresis and visualized by exposure to ultra violet light (302 nm) after red stain staining. Sequencing of gene was performed by national instrumentation center for environmental management (nicem) online at (http://nicem.snu.ac.kr/main/?en_skin=index.html), biotechnology lab, machine is DNA sequencer 3730XL, Applied Biosystem), Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at ([http:// www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and BioEdit program.

2.6. Statistical Analysis:

The data were expressed as mean $\pm$ SD, and the probability was expressed by using Duncan test the statistical analysis program IBM SPSS version 24, Pearson's chi-square Fischer exact probability and odd ratio were used to expressed the significance between the studied groups for polymorphisms and related parameters by using the program WinPepi version 11.65, while for genotyping and allele frequency calculation, the online Hardy-Weinberg equilibrium calculator was used.



CHAPTER THREE

RESULTS AND DISCUSSION

3.1. Clinical and Characteristic Features of the Studies Groups:

Sixty eight females with unexplained infertility (UI) were included in this study. They have been distributed depending on the type of infertility into two groups, primary (1°UI) and secondary (2°UI) infertility. The groups of females with unexplained infertility were compared with groups of females with poly cystic ovarian syndrome infertility PCOS (sixty eight women with PCOS), which was also distributed depending on the type of infertility into two groups primary (1°PCO) and secondary (2°PCO) infertility. All groups of infertile women were compared with group of apparently healthy fertile control (thirty four women) without significant difference in Age, as shown in Table (3-1).

Table (3-1): Characteristic data for study subjects.

Groups	NO.	Age year	BMI(Kg/m ²)
		<i>mean± SD</i>	<i>mean± SD</i>
Control (fertile woman)	34	32.28±6.70	21.13± 3.38
1° UI	34	29.25 ± 6.26	22.45±4.55
2° UI	34	33.69 ±6.36	22.39±3.89
1° PCO	34	25.844 ± 4.47	24.36±5.45
2° PCO	34	28.44± 4.21	24.66±5.66

3.2 Biochemical study For Women with Unexplained Infertility:

3.2.1 General Comparison:

3.2.1.1 Serum Hormones Concentration:

Table (3.2) and figures (3.1), (3.2), (3.3) and (3.4) show non significant differences ($P>0.05$) in PRL, TSH, FSH and LH levels in the two groups of women with (1°UI) and (2°UI) in comparison with the control group. These results were compatible with (Beyazit *et al.*, 2016; Verit *et al.*, 2017). Also the study was found non significant differences ($p>0.05$) in such hormones levels between the two groups of the women with UI, can be observed.

Same table and figures (3.1), (3.2), (3.3) shows a significant increase in PRL, TSH and LH hormone levels in the two groups of women with PCOS infertility (1°PCO) and (2°PCO) in comparison with the control group ($p\leq 0.05$). These results agreed with the results reported by (George and Malini, 2014; Shah *et al.*, 2017). Also the current study shows a significant increase in PRL, TSH and LH hormone levels in the two groups of women with (PCOS) in comparison with the two groups of women with (UI) respectively ($p\leq 0.05$). This result agrees with the study of (Diamond *et al.*, 2017), while there are no significant differences in PRL, TSH and LH hormone levels between the two groups of the women with (PCOS) infertility. These results are matched with the results of the study of (Spandana *et al.*, 2017).

Futhermore the results showed no significant differences in FSH hormone levels in the (1°UI, 2°UI, 1°PCO and 2°PCO) in comparison with control group, and also there are no significant differences in FSH hormone levels in the two groups of the women with unexplained infertility (1UI) and (2°UI) in comparison with the two groups of the women with PCOS infertility

(1°PCO) and (2°PCO) respectively. These results are agreed with studies of (Beyazit *et al.*, 2016; Mohammed *et al.*, 2016). Moreover, no significant differences in FSH hormone levels between the two groups of women with (UI) and (PCOS) can be observed.

Hyperprolactinemia can cause symptoms similar to PCOS. Reports have suggested that women with PCOS have elevated prolactin levels. This increased prolactin may augment by the inhibition of 3- β -hydroxysteroid dehydrogenase activity or, less often, through selective action on the sulfation of dehydroepiandrosterone DHEA in adrenal or extra-adrenal sites (George and malini, 2014).

It is observed that the prevalence of hyperprolactinemia associated hypothyroidism which is characterized by high levels of serum TSH and low levels of thyroid hormones (T3 and T4). Infertility due to hyperprolactinemia has been found to be associated with abnormal menstrual pattern as well as anovulatory cycles. (Valvekar and Lakshmi, 2016).

In many infertility cases, the diagnosis is simply unexplained because a variety of reasons like lack of ovulation, mechanical stoppage and parental age etc. (Kumar *et al.*, 2014). The ovulation can be affected by hormonal imbalance, thyroid disorders or chronic diseases such as diabetes (Sultana and Nadir 2008). Some women with hyperprolactinemia might have primary hypothyroidism (Valvekar *et al.*, 2016).

Abnormality of the hypothalamic-pituitary ovarian or adrenal axis has been implicated in PCOS. Disturbance in the pulsatility of gonadotrophin releasing hormone (GnRH) results in the relative increase in LH to FSH release. An abnormal feedback mechanism by ovarian estrogen is blamed to

play role in this discriminated increase in LH release. (Najem *et al.*, 2008; Spandana *et al.*, 2017).

Table (3 – 2):- Serum Hormones levels of control and infertile groups

Groups	NO.	PRL (μU/ml)	TSH (μU/ml)	LH (μU/ml)	FSH(μU/ml)
Control	34	14.5±3.9 ^b	2.3±0.7 ^b	3.2±0.9 ^{bc}	5.4±1.4 ^a
1° UI	34	14.5 ±2.9 ^b	2.3±0.7 ^b	2.6±0.7 ^c	5.3±1.2 ^a
2° UI	34	14.4 ±2.9 ^b	2.1± 0.6 ^b	3.6±1.0 ^b	5.4±1.4 ^a
1° (PCO)	34	16.3 ± 3.0 ^a	2.8±0.7 ^a	8.1±1.8 ^a	5.3±1.3 ^a
2° (PCO)	34	16.1 ±3.0 ^a	2.9 ± 0.8 ^a	8.0±1.7 ^a	5.4±1.4 ^a

- Each value represents mean ± S.D values with non-identical superscript (a, b or c ...etc.) were considered similar letters: No significant difference ($p > 0.05$) between means, different letters: significant difference ($p \leq 0.05$) between means

Control: fertile woman

1° UI: Primary unexplained infertility

2° UI: Secondary unexplained infertility

1° PCO: Primary explained infertility

2° PCO: Secondary explained infertility

-No: Number of subjects.

-SD: Standard deviation.

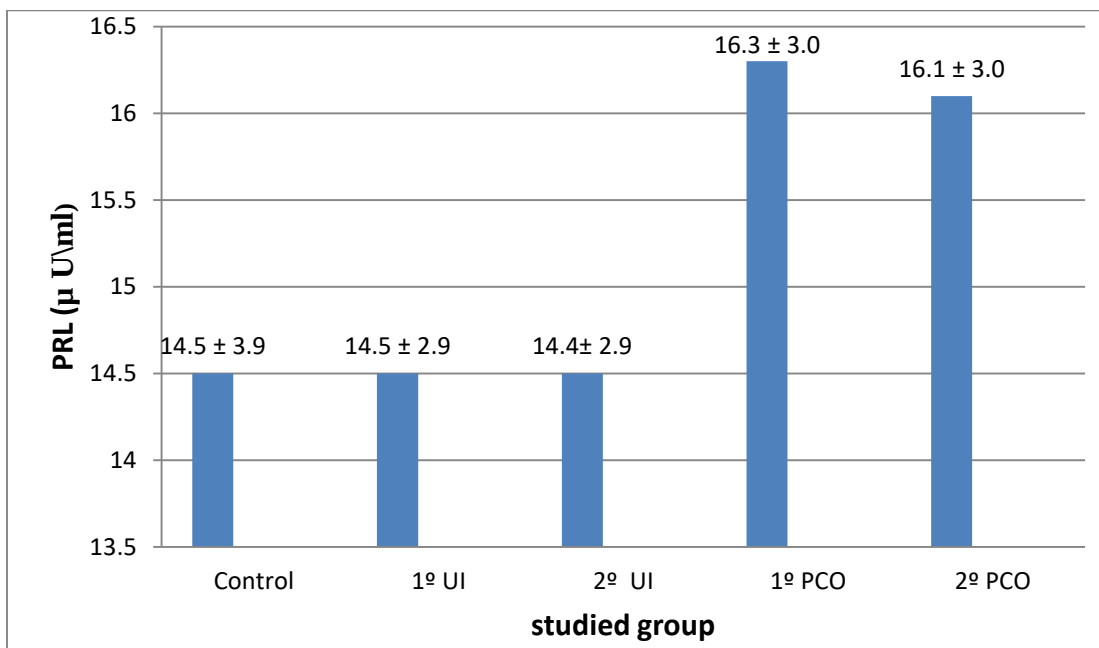


Figure (3.1): Serum PRL levels in control and infertile women groups

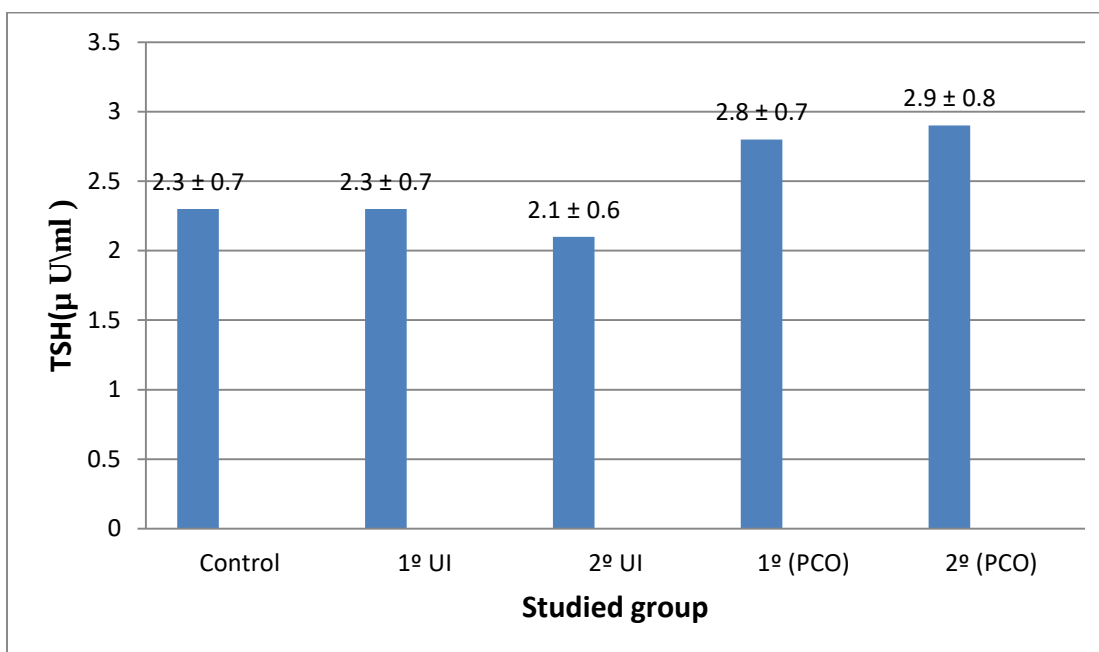


Figure (3.2): Serum TSH levels in control and infertile women groups

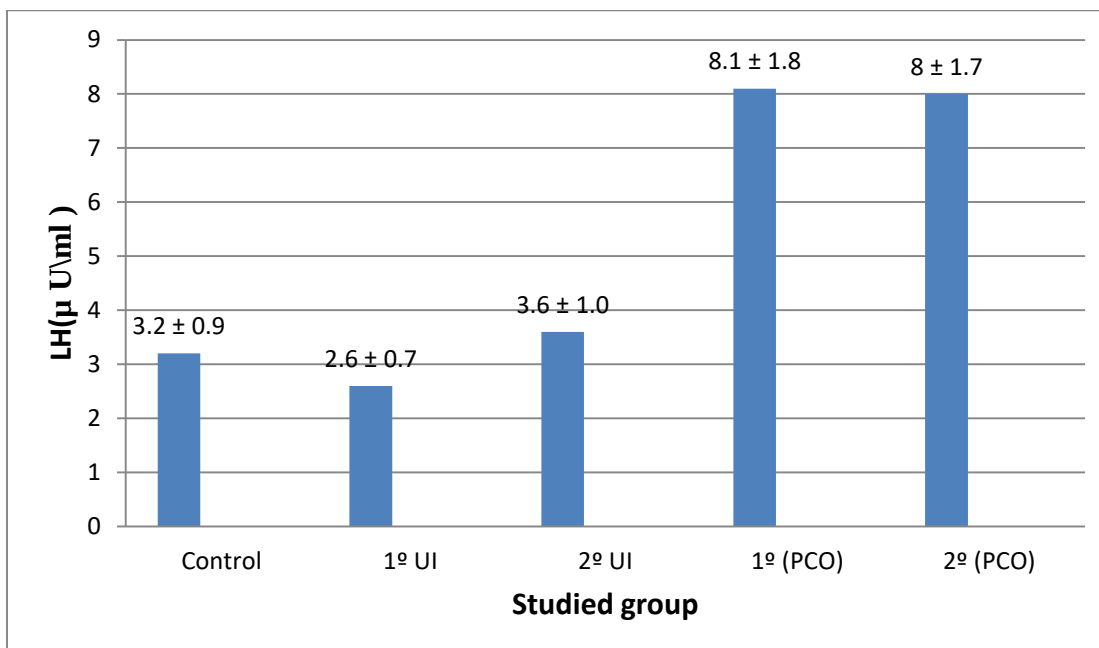


Figure (3.3): Serum LH levels in control and infertile women groups

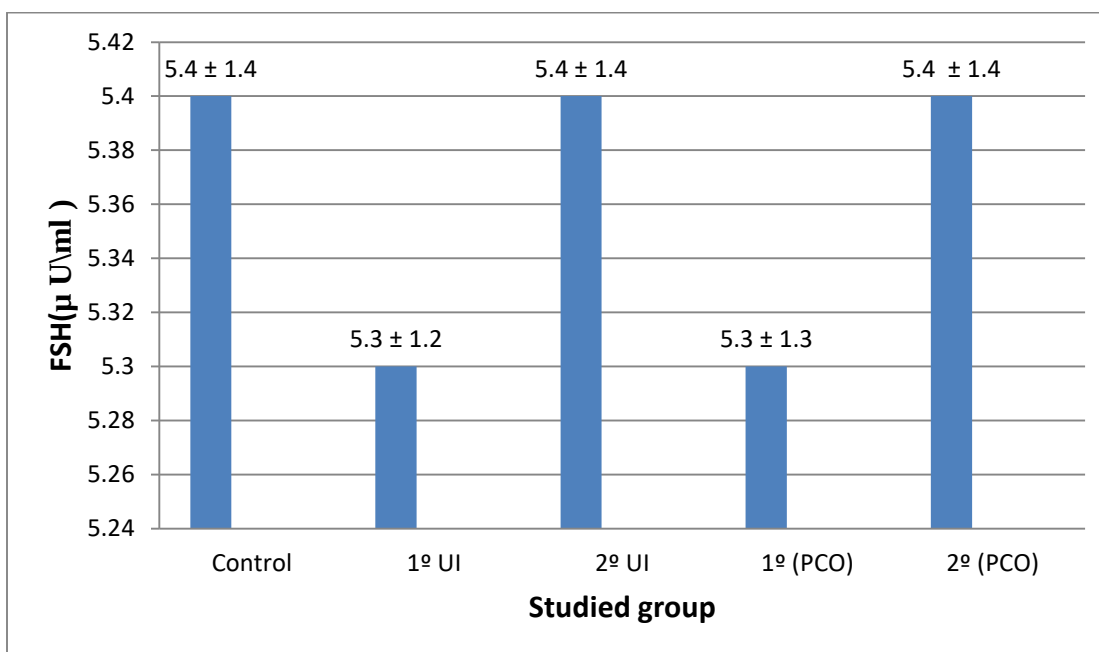


Figure (3.4): Serum FSH levels in control and infertile women groups

3.2.1.2 Serum Malondialdehyde (MDA) Concentration:

The results showed a significant increase in the concentration of serum MDA in all women with unexplained infertility (UI) and (PCOS) in comparison with control groups ($p \leq 0.05$). These results are similar to the results of (Soleimani *et al.*, 2015).

Table (3-3) and figure (3-5) shows a significant increase ($p \leq 0.05$) in the concentration of serum MDA in the two groups of woman with unexplained infertility (UI) in comparison with control. These results are compatible with the studies of (Savita *et al.*, 2009; Al-Mukhtar *et al.*, 2012; ALL-Ahmed *et al.*, 2015). However, there was no significant difference ($p > 0.05$) in the concentration of serum MDA between the two groups of woman with unexplained infertility (1°UI) and (2°UI). These results agreed with the results of the study of (Al-Mukhtar *et al.*, 2012). On the other hand, our findings referred to a significant increase ($p \leq 0.05$) in the concentration of serum MDA in the two groups of woman (1°PCO) and (2°PCO) in comparison with control. These results are compatible with the studies of (Deepika *et al.*, 2014; Sumithra *et al.*, 2015; Zahoorunnisa *et al.*, 2017). Also there was a significant increase ($p \leq 0.05$) in the two groups of women (1°PCO) and (2°PCO) in comparison with the two groups of woman with unexplained infertility (1°UI) and (2°UI) respectively. These results are in agreement with results of the study of (Diamond *et al.*, 2017).

In the human body, reactive oxygen species (ROS) are formed under physiologic and pathologic condition (Ruder *et al.*, 2008). They can be produced from endogenous sources, for instance during aerobic metabolism and due to different metabolic pathways, or as part of defense mechanism of the body (Agarwal *et al.*, 2008). In addition, ROS can be form source

exogenously such as numerous environmental pollutants, and by cigarette and alcohol drinking (Ruder *et al.*, 2008). Data have been implicated that regulated levels of ROS in ovaries, endometrium, fallopian tube, embryos, and peritoneal fluid play a role in tissue remodeling, hormone signaling, ovarian steroidogenesis, folliculogenesis, maturation of oocyte, tubal function, and cyclical and endometrial changes (Agarwal *et al.*, 2005). However, pathologic levels of ROS. have resulted in considerable damage to cell structure (Polak *et al.*, 2001). Moreover, some evidence reported that ROS are involved in developing female infertility (Ruder *et al.*, 2008; Visioli and Hagen, 2011).

There are many factors that have relationship of ROS and reproduction in female such as: Maternal age, oocyte quality decreases when increasing maternal age. The low quality of oocytes contains increased mitochondrial DNA damage and chromosomal aneuploidy, secondary to age-related dysfunctions. These mitochondrial changes may arise from elevated ROS, which occurs through the opening of ion channels (e.g. loss of Ca^{2+} homeostasis) (Ruder *et al.*, 2009). Menses stopping cause increase iron levels in body which lead to elevated irons stores in body and could be induce oxidative imbalance (Tropea *et al.*, 2006).The high rate of free radicals production in females with unexplained infertility may be generated from increases metabolism and depletion of protective antioxidants (Al.Mukhtar *et al.*, 2012).

Obesity is considered one of some factors leading to the more serious oxidative status of PCOS. Insulin resistance (IR) also encourages OS because hyperglycemia and higher levels of free fatty acid lead to reactive oxygen species (ROS) production (Lee *et al.*, 2010). When excess glucose or free fatty acid are absorbed in the cell, a large number of reducing

metabolites, just like pyruvic acid and acetyl coenzyme A, will be transferred into mitochondria for oxidization, leading to enhancing the activity of electron transport chain and single electron transfer, finally resulting in increasing ROS production. Furthermore, oxidative stress would be caused if reducing enzymes, just like super oxide dismutase (SOD), peroxidase, and catalase, fails to scavenge the excess ROS in the cell (Pisoschi and Pop, 2015). The lipid peroxidation in women with PCO evaluated in comparison with women with UI may be due to the effects of Obesity, hyperandrogenemia and Insulin resistance.

Table (3 – 3): Serum MDA levels of control and patients groups.

Groups	NO.	MDA(μmol/L) <i>mean</i> $\pm$ <i>SD</i>
Control	34	2.01 $\pm$ 0.44 ^c
1° UI	34	3.0 $\pm$ 0.58 ^b
2° UI	34	3.0 $\pm$ 0.57 ^b
1° PCO	34	3.5 $\pm$ 1.1 ^a
2° PCO	34	3.8 $\pm$ 1.1 ^a

- Legend as in table (3.2)

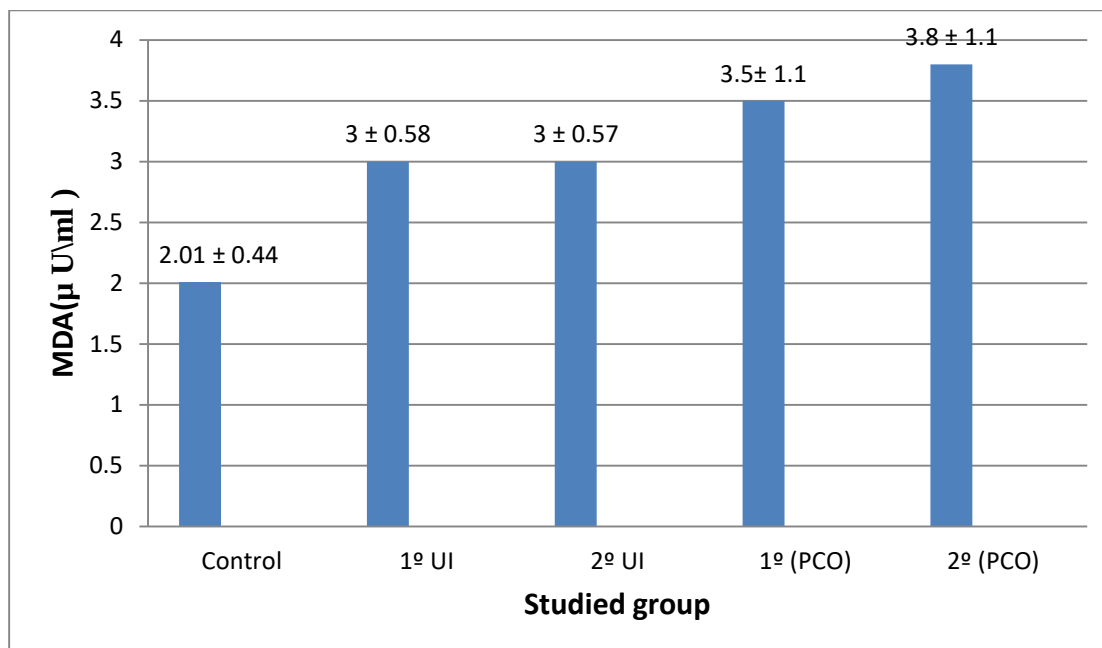


Figure (3-5): Serum MDA levels of control and fertile women groups

3.2.1.3. Serum Antioxidant Concentrations:

Table (3-4) and figure (3-6) show a significant decrease ($p \leq 0.05$) in the concentration of serum GSH in the two groups of the women with unexplained infertility (1°UI) and (2°UI) in comparison with the control group. These results are in agreement with the study of (Al-Mukhtar *et al.*, 2012 and ALL-Ahmed *et al.*, 2015). Also there is a significant decrease ($p < 0.05$) in concentration of serum GSH in (2°UI) group in comparison with (1° UI), (1° PCO) and (2° PCO) groups respectively.

The same table also showed a significant decrease ($p \leq 0.05$) in the concentration of serum GSH in the two groups of women (1° PCO) and (2° PCO) in comparison with the control group. These results are compatible with the results of the studies of (Kurdoglu *et al.*, 2012 and Murri *et al.*, 2013 and Santos *et al.*, 2016), However there are no significant differences

($p > 0.05$) in the concentration of serum GSH between (1° UI), (1° PCO) and (2° PCO) respectively, can be observed.

The table (3-3) and figure (3-7) show a significant decrease ($p < 0.001$) in the concentration of serum (SOD) in (2°UI) and (2°UI) groups in comparison with the control group. These results are matched the results of the studies of (Al.Mukhtar *et al.*, 2012 and ALL-Ahmed *et al.*, 2015). Also there is a significant decrease ($p \leq 0.05$) in concentration of serum (SOD) in (1° PCO) and (2° PCO) groups in comparison with the control group. These results are compatible with the results of the studies of (Santos *et al.*, 2016; Kolesnikova *et al.*, 2016. However there are no significant differences ($p > 0.05$) in concentration of serum (SOD) among (1°UI) and (2°UI) as well as (1° PCO) and (2° PCO) groups respectively, can be observed

The results showed a significant decrease ($p \leq 0.05$) in the concentration of serum Cp in (1°UI) and (2°UI) women groups in comparison with the control groups. However no significant differences ($p > 0.05$) in concentration of serum ceruloplasmin between (1°UI) and (2°UI) groups can be observed. Whereas there was a significant decrease ($p \leq 0.05$) in the concentration of serum Cp in (1° PCO) and (2° PCO) groups comparison with (1°UI) and (2°UI) groups.

The low serum GSH and SOD concentration in women with UI in comparison with fertile women in this study may be due to elevated ROS levels in patients with unexplained infertility implies exhausted antioxidant defense, resulting in the inability to scavenge ROS and neutralize their toxic effects (Wang *et al.*, 1997). Many studies have been found positive relationship between excess oxidative stress and decrease the antioxidant enzymes activities which lead to impaired infertility in females (Combelles *et al.*, 2009; Victor *et al.*, 2011). Normally oocytes grow and developed in

ovary every month, but first meiosis resumes in one only of them (dominant oocyte). This process cause elevated in ROS levels and inhibition by antioxidant. While progression of meiosis II is promoted by antioxidants (Agarwal *et al.*, 2012). Behrman *et al.*, has been reported a presence of complication relationship between ROS and antioxidants in ovary (Behrman *et al.*, 2001). Elevated production of steroid in follicle growing lead to increase cytochrome P450 enzyme, resulting formation of ROS, which produced by pre-ovulatory follicle have important role to inducer ovulation process. (Ruder *et al.*, 2009). The oxygen which considered important for growth and development of ovarian follicles by prevent stimulation follicle angiogenesis. ROS Follicles raise apoptosis, while Glutathione (GSH) and follicular stimulating hormone (FSH) necessary for growing follicle. Increases in the levels of estrogen as results to FSH stimulation, triggering the generation of catalase in the dominant follicle, and thus avoiding apoptosis (Behrman *et al.*, 2001).

In addition, spermatozoa and oocyte have built in a mechanism to prevent excessive production of ROS at the time of sperm-oocyte fusion. This may be done by the release of antioxidant enzymes (Maiorino and Ursini, 2002), and if there is an abnormality in the production of antioxidant enzymes, ROS generation can continue uninterruptedly and damage both spermatozoa and oocyte (Aitken *at al.*, 2003).

The decrease in GSH and SOD levels in women with (PCOS) in comparison with fertile women may be due to the pathogenesis of PCOS that is complex and its underlying basis remains unclear. Several characteristics and associations of PCOS, including androgen excess, abdominal adiposity, insulin resistance and obesity, may contribute to the development of local and systemic oxidative stress (Liu *et al.*, 2010; Nasiri

et al., 2015), which may reciprocally worsen these metabolic abnormalities, therefore the women with PCOS infertility have reduced concentrations of antioxidants and increased ROS-induced lipid peroxidation damage resulting in infertility (Shirsath *et al.*, 2015). Indeed, a reduction in the GSH/ GSSG ratio and GSH levels occurs due to the increase in ROS production and a decrease in oxygen consumption which results in impaired mitochondrial function in PCOS women (Dincer *et al.*, 2005).

The low level of serum ceruloplasmin in infertile women may be due to its role in the as a ferroxidase which is of greatest importance as it converts reduced (ferrous) linked with transferrin to oxidized (ferric) iron linked with ferritin. Fe^{+2} acts as pro-oxidant agent because of its readiness to change from one valency state to another. In its free form, iron is one of the most effective antioxidant catalysts (Philippe *et al.*, 1988). Furthermore, Iron accumulation in liver and increase copper concentration may be cause decrease in ceruloplasmin level in blood (Ganz and Nemeth 2015). This result is in disagreement with the study of Al-Helaly and Al-Kado on infertile females in which they showed that higher levels of ceruloplasmin was in infertile women rather than in the control group (Al-Kado and Al-Helaly, 2012).

Ceruloplasmin constitutes a significant part of extracellular antioxidant defense system with having both antioxidant and pro-oxidant effects in tissues (Tapiero *et al.*, 2003; Uriu-Adams and Keen 2005). The low serum ceruloplasmin levels in women with unexplained infertility which supports the hypothesis that low antioxidant levels are linked to the pathophysiological aspects of unexplained infertility. Ceruloplasmin, beside its prooxidant activity, behaves as a potent antioxidant for various oxygen

radicals collecting oxygen derived free radicals in the blood stream (Atanasiu *et al.*, 1998 ; Healy and Tipton, 2007).

Table (3 – 4):- Serum antioxidant levels of control and fertile women groups

Groups	NO.	GSH ($\mu\text{mol/L}$) <i>mean</i> $\pm$ <i>SD</i>	SOD (U/mg) <i>mean</i> $\pm$ <i>SD</i>	CP (<i>g/l</i>) <i>mean</i> $\pm$ <i>SD</i>
Control	34	524.2 $\pm$ 132.2 ^a	5.4 $\pm$ 2.1 ^a	3.7 $\pm$ 0.9 ^a
1 ^o UI	34	305.4 $\pm$ 82.5 ^b	3.0 $\pm$ 1.2 ^b	2.9 $\pm$ 0.9 ^b
2 ^o UI	34	248.4 $\pm$ 88.3 ^c	3.3 $\pm$ 1.3 ^b	3.0 $\pm$ 0.9 ^b
1 ^o PCO	34	277.0 $\pm$ 92.8 ^{bc}	3.2 $\pm$ 1.3 ^b	1.4 $\pm$ 0.4 ^c
2 ^o PCO	34	274.3 $\pm$ 88.2 ^b	3.0 $\pm$ 1.2 ^b	1.4 $\pm$ 0.4 ^c

- Legend as in table (3.2)

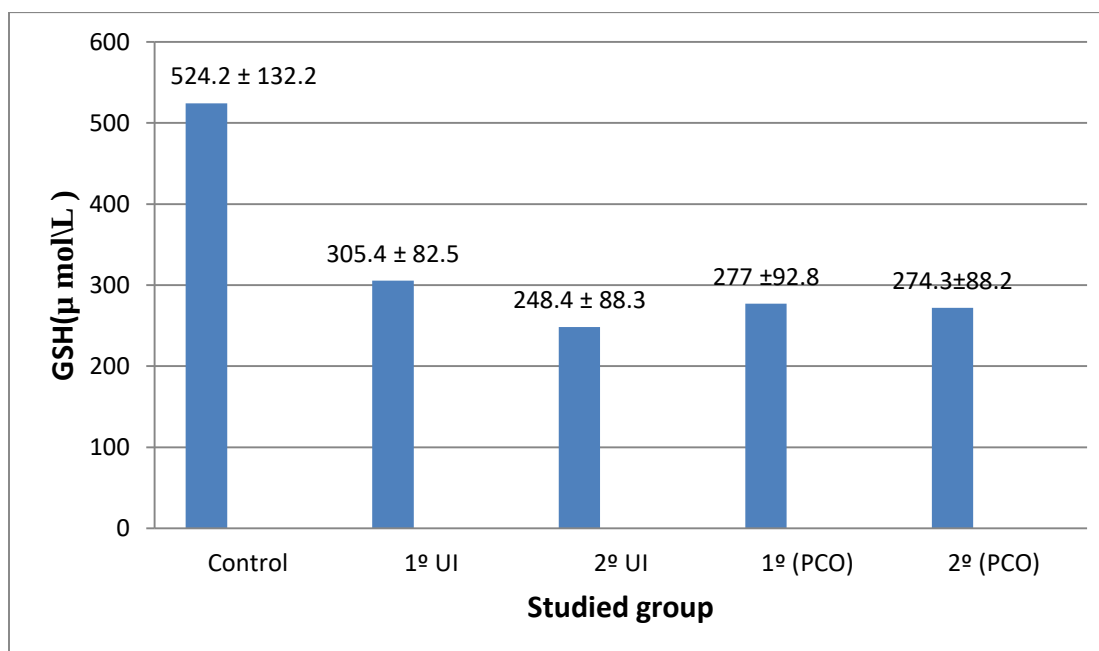


Figure (3.6): Serum GSH levels of control and infertile women groups

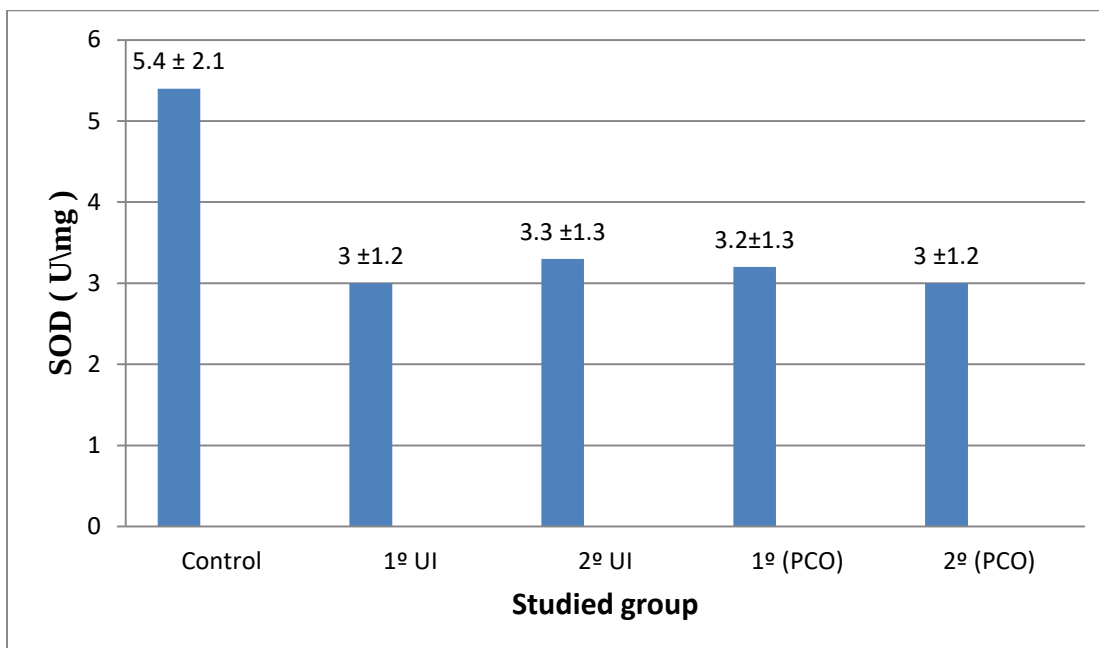


Figure (3.7): Serum SOD levels of control and infertile women groups

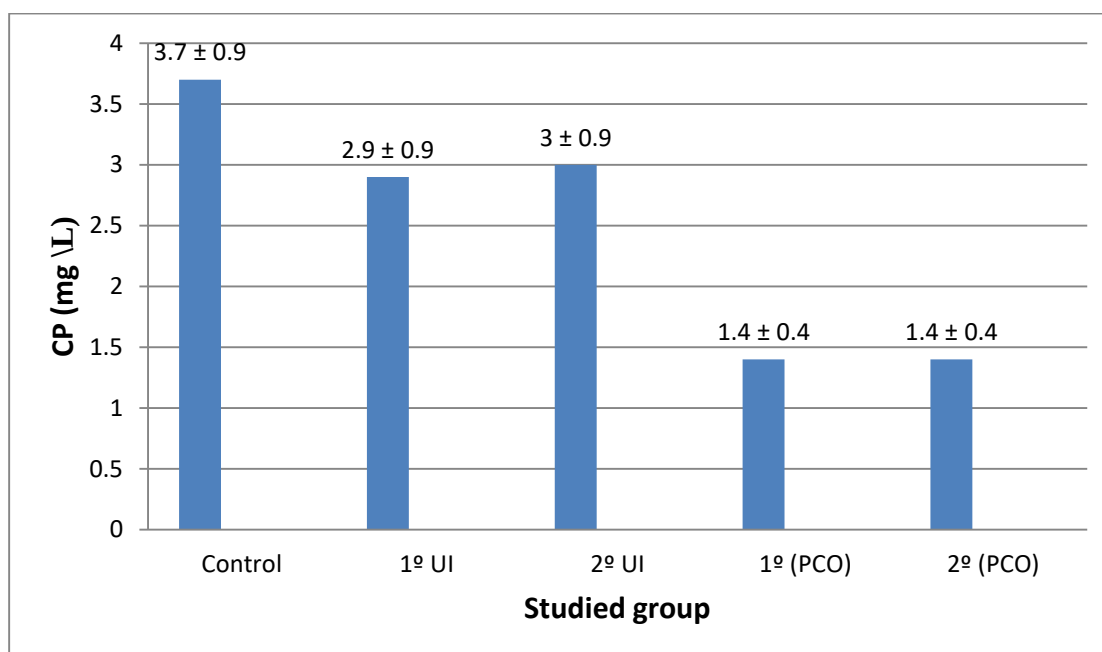


Figure (3-8): Serum CP levels of control and infertile women groups

3.2.1.4. Serum Lipid Profile Concentrations:

Table (3-5) and figure (3-9), (3-10) and (3-11) show a significant increase ($p \leq 0.05$) in the concentration of serum (TC) and (LDL) in (1°UI) and (2°UI) groups as well as a significant decrease ($p \leq 0.05$) in (2°UI) group in the concentration of serum (HDL) in comparison with the control group. These results are compatible with results of the study of (Verit *et al.*, 2016). There is a significant increase ($p \leq 0.05$) in the concentration of serum (TC) in (2°UI) group in comparison with (1°UI) group, while there are no significant differences ($p > 0.05$) in the concentration of serum (LDL) between the two groups of women with (UI). Also the results show a significant decrease ($p \leq 0.05$) in the concentration of serum (HDL) in (1°UI) group in comparison with (2°UI), (1°PCO) and (2°PCO) groups respectively.

The same table shows a significant increase ($p \leq 0.05$) in the concentration of serum (TC) and (LDL) and a significant decrease ($p \leq 0.05$) in the concentration of serum (HDL) in the two groups (1°PCO) and (2°PCO) in comparison with the control group. These results are compatible with the results of the studies of (Latha *et al.*, 2012; George and Malini, 2014 ; Shah *et al.*, 2017). Also, it is a significant increase ($p \leq 0.05$) in the concentration of serum (TC) and (LDL) in the two groups of women (1°PCO) and (2°PCO) in comparison with the two groups of women with unexplained infertility (1°UI) and (2°UI) respectively. These results are matched with the study of (Michael *et al.*, 2017); however there are no significant differences ($p > 0.05$) in the concentration of serum cholesterol and (LDL) between (1°PCO) and (2°PCO) groups. Also the results show non significant differences ($p > 0.05$) in the concentration of serum (HDL) among (2° UI), (1°PCO) and (2°PCO) groups.

The same table and figures (3-12, 3-13) show a significant increase ($p < 0.05$) in the concentration of serum triglyceride (TG) and VLDL in the two groups of unexplained infertile women (1°UI) and (2°UI) in comparison with the control group. This result is agreed with the study of (Verit *et al.*, 2017). The study shows non significant differences ($p > 0.05$) in the concentration of serum (TG) and VLDL between the two groups of unexplained infertile women (1°UI) and (2°UI). Also, there is a significant increase ($p \leq 0.05$) in the concentration of serum (TG) and VLDL in the two groups of women (1°PCO) and (2°PCO) in comparison with the control group, these results are agree with the results reported by (Latha *et al.*, 2012, Mohammed *et al.*, 2017, Sharif *et al.*, 2017). Furthermore there is a significant increase ($p \leq 0.05$) in the concentration of serum triglyceride (TG) and VLDL in the two groups (1°PCO) and (2°PCO) in comparison with (1°UI) and (2°UI) groups. However no significant differences ($p > 0.05$) in the concentration of serum triglyceride and VLDL between the two groups of women (1°PCO) and (2°PCO), can be observed.

Elevation level of lipid profile and decrease level of HDL in infertile women was may be due to life style, genetic factors and this elevation is a risk factor of coronary heart diseases. It is widely known that infertility has been suggested to be a risk factor for cardiovascular disease CVD (Gregg *et al.*, 2005). The structure and abundance of the circulating HDL to which the ovaries are exposed. This evidence also suggests that the infertility is likely caused by defects in oocytes/ eggs manifested during the periovulatory period without affecting the primordial follicle pool. Indeed, ~19% of the ovulated eggs harvested ~16 h after hormone-induced superovulation are

dead (Yesilaltay *et al.*, 2014). The remaining oocytes are unable to be fertilized or develop into viable pups.

Endometriosis, another common cause of unexplained infertility, is characterized by dyslipidemia, local and systemic chronic inflammation and oxidative stress (Verit *et al.*, 2008). Many studies have shown that dyslipidemia, aging, obesity and chronic inflammation are related with reduced oocyte quality and quantity (Wellons, 2011; Michalakis and Coppack, 2012).

Elevated body mass index, total cholesterol, TG, LDL-c levels and low level of HDL-c are associated with the risk for the development of metabolic syndrome (Sujata, 2013). This study showed deranged lipid profile in PCOS women with the findings of elevated TC, TG, VLDL-c and LDL-c levels and low HDL-c levels supporting the existing knowledge of the studies of Vinita and Monini, (2017); Kumar *et al.* (2016) and Naidu *et al.* (2017) which indicated that dyslipidemia is prevalent in women with PCOS. The present study showed significantly raised cholesterol and LDL in PCOS women and is similar to the findings of Thathapudi Sujatha *et al.* (2014) but contradictory to the findings of Rasool Suzan Omer. (2015).

A 50% increased risk of myocardial infarction or coronary heart disease was found among women with menstrual irregularities in comparison with those who had normal cycles from the ages of 20 to 35 years. Obesity, insulin resistance, hypothyroidism, anovulation, and CVD are also related with infertility (Flynn *et al.*, 2006; Verit *et al.*, 2016)

Furthermore women with PCOS would be predicted to be at high risk for dyslipidemia because they have elevated androgen levels and are frequently obese. PCOS women are often hyperinsulinemic and insulin resistant, which can lead to dyslipidemia (Latha *et al.*, 2012). Progressive elimination of lipid

and apolipoproteins from the VLDL particle leads to an increased formation of intermediate-density and low-density lipoproteins, both of which are atherogenic (DeFronzo and Ferrannini, 1991)

If the concentration of VLDL-transported triglyceride is high, cholesteryl ester transfer protein CETP promotes the transfer of LDL cholesteryl ester or HDL cholesteryl ester in exchange for triglyceride. Triglyceride-rich HDL cholesterol or LDL cholesterol can undergo hydrolysis by hepatic lipase or lipoprotein lipase (Fenkci *et al.*, 2003)

Table (3-5): Serum lipid profile levels of control and fertile women groups.

Groups	No	TC mg/dl <i>mean± SD</i>	LDL mg/dl <i>mean± SD</i>	HDL mg/dl <i>mean± SD</i>	TG mg/dl <i>mean± SD</i>	VLDL mg/dl <i>mean± SD</i>
control	34	139.0±29.2 ^d	72.7 ± 21.3 ^c	49.0±14.6 ^a	99.2±23.9 ^c	18.2±5.4 ^c
1° UI	34	146.9±29.3 ^c	91.6±23.4 ^b	34.9± 8.2 ^c	102.0±30.7 ^b	20.7±6.1 ^b
2° UI	34	154.7±29.5 ^b	91.4±25.0 ^b	40.7± 8.5 ^b	112.7±27. 7 ^b	23.1±5.9 ^b
1° PCO	34	166.7±32.9 ^a	101.8±23.4 ^a	43.6± 8.3 ^b	129. 0±37.3 ^a	25.9±7.5 ^a
2° PCO	34	164.8±32.0 ^a	101.8±23.4 ^a	42.2 ± 9.7 ^b	130.6±36.6 ^a	26.2±7.3 ^a

-Legend as in Table (3.2)

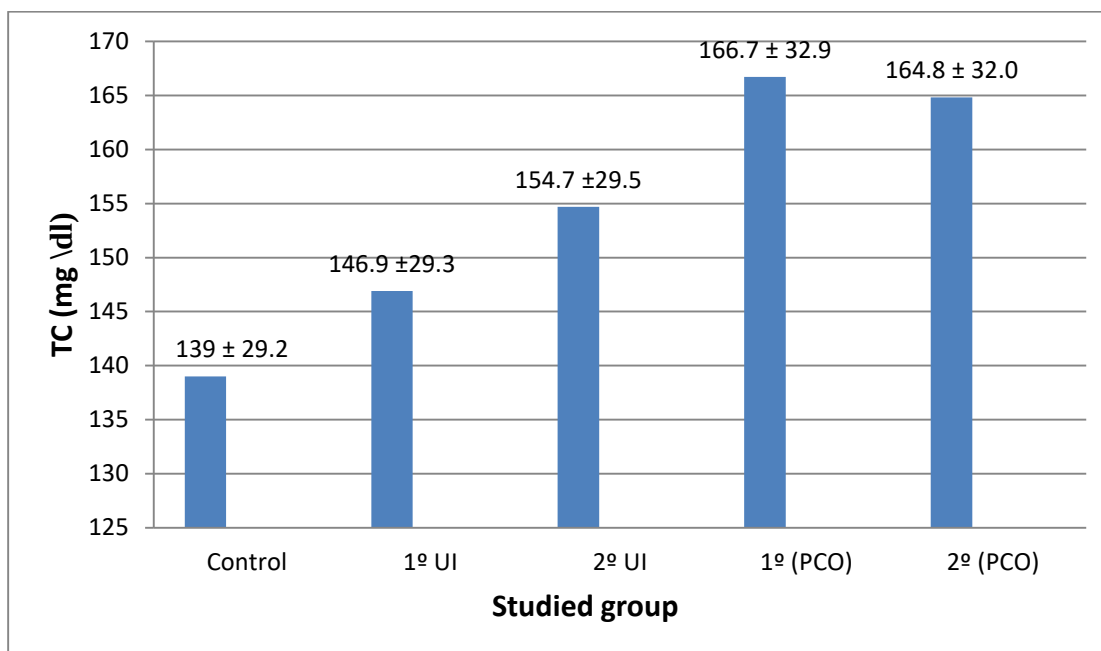


Figure (3-9): Serum TC levels of control and infertile women groups

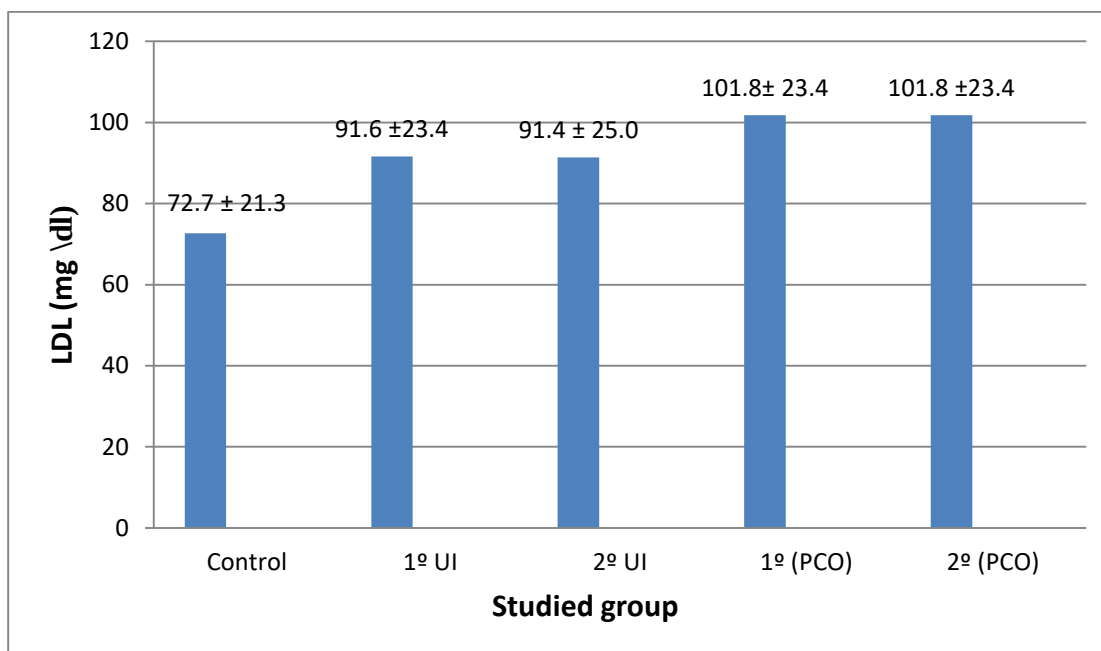


Figure (3-10): Serum LDL levels of control and infertile women groups

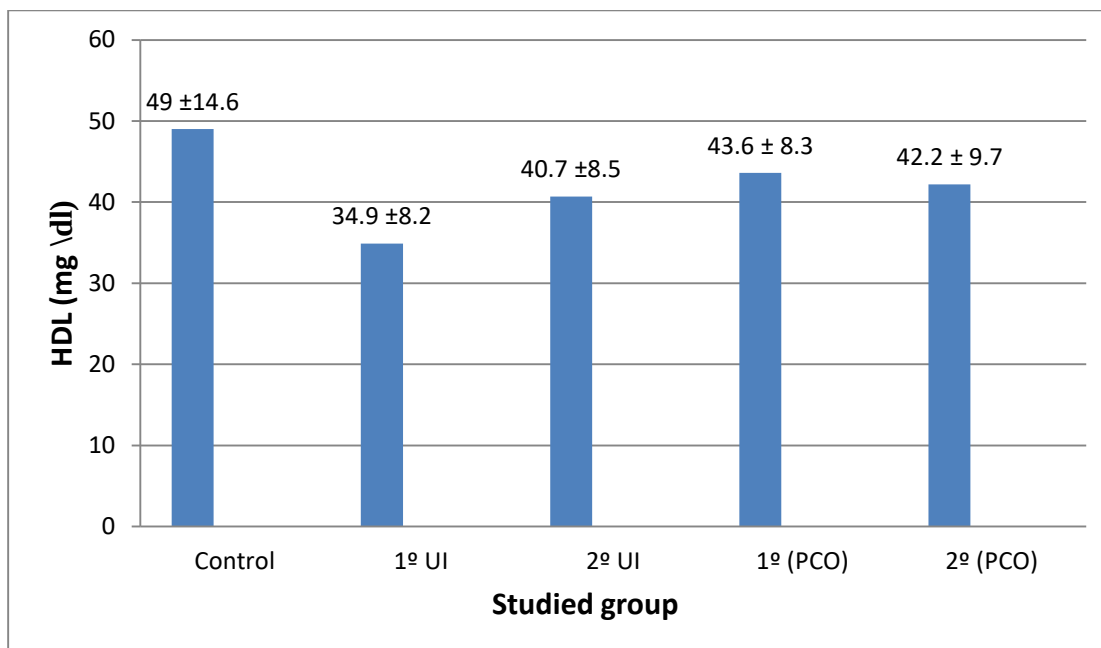


Figure (3-11): Serum HDL levels of control and infertile women groups

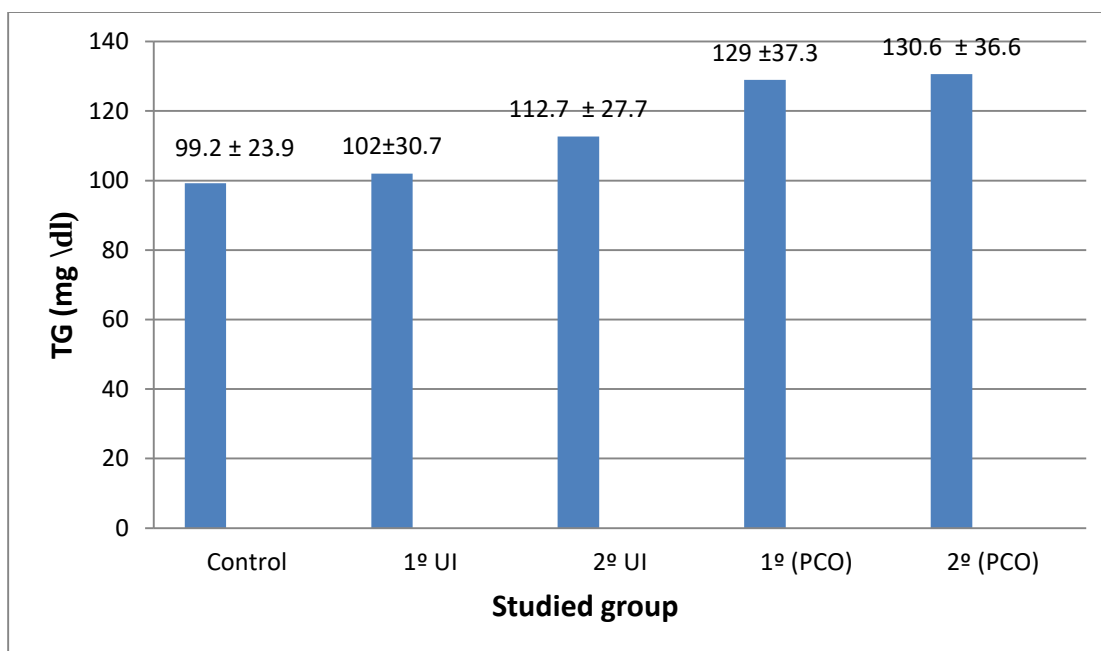


Figure (3-12): Serum TG levels of control and infertile women groups

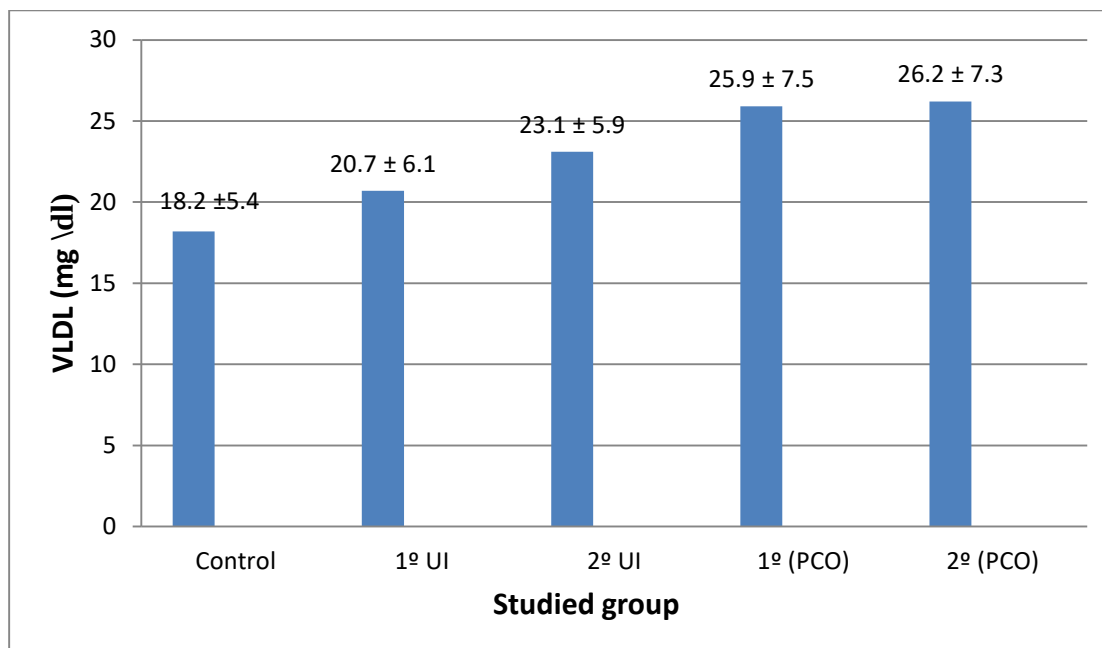


Figure (3-13): Serum VLDL levels of control and infertile women groups

3.2.1.5. Serum Trace Elements Concentration:

The Table (3.6) and figure (3.14) show a significant increase ($p \leq 0.05$) in the concentration of serum iron in the two groups of women with unexplained infertility (1°UI) and (2°UI) in comparison with the control group. The same table shows a significant increase ($p \leq 0.05$) in the concentration of serum iron in (1°UI) group in comparison with (2°UI), (1°PCOS) and (2°PCO) groups. This result is in disagreement with the results of the study of (Alwais *et al.*, 2016). Whereas non significant differences ($p > 0.05$) in the concentration of serum iron in (1°PCOS) and (2°PCO) groups in comparison with the control group, can be observed. This result matched with the result of the study of (Li *et al.*, 2017; RashidiShariat *et al.*, 2017).

Excess iron may be leads to reduced production of the hormones LH and FSH from the anterior pituitary suggesting impaired oocyte maturation and low ovarian reserve (Singer *et al.*, 2011). There are few studies investigating the effect of iron overload (toxicity) and fertility and even less with regard to female fertility. However, there is a link between iron overload and female infertility (Mishra and Tiwari, 2013).

In this study, no significant differences in serum levels of iron can observed between each groups of women with (PCOS) and control group. A research conducted by Taghavi *et al.* in 2009 in Iran on 50 PCOS patients without overweight did not show iron overload in these patients of (PCOS) (Taghavi *et al.*, 2009). A cross-sectional study of Li *et al.* on 578 people in 2016 indicated that the serum levels of iron had no difference between the two PCOS and non-PCOS groups (Li *et al.*, 2017).

In addition, both iron and glucose metabolism may be cause of oxidative stress, which is involved in the pathogenesis of many complex metabolic disorders including PCOS (Moyer *et al.*, 2011; Escobar-Morreale *et al.*, 2011). Free iron is highly toxic and participates, through the Fenton reaction, in the formation of free radicals including hydroxide and superoxide anions, thereby contributing to oxidative stress (Fernández-Real *et al.*, 2002). Moreover, inflammatory cytokines increase transferrin receptors on the cell surface favoring iron deposition in tissues, and are as well intimately involved in the development of obesity associated insulin resistance (Fernandez-Real and Ricart, 2003).

Table (3-6) and figure (3-15) show a significant increase ($p \leq 0.05$) in the concentration of serum copper in the two groups of the women with unexplained infertility (1° UI) and (2° UI) in comparison with the control group. These results are matched with the results of the study of (AL-Saraf,

2005). Also there is a significant increase ($p \leq 0.05$) in the concentration of serum Cu in the two groups of the women with unexplained infertility (1°UI) and (2°UI) in comparison with the two groups of women with (1°PCO) and (2°PCO) groups. While there are no significant differences ($p > 0.05$) in the concentration of serum Cu between the two groups of the women with unexplained infertility, can be observed.

This study shows a significant increase ($p \leq 0.05$) in the concentration of serum Cu in the two groups of women with (PCOS) in comparison with the control group. This result matched with the results of the studies of (Kurdoglu *et al.*, 2012; Muyan *et al.*, 2016). Whereas there are non significant differences ($p > 0.05$) in the concentration of serum Cu between the two groups of the women with (PCOS) infertility.

This elevation in serum copper levels in women with (UI) may directly affect infertility rates by lowering progesterone levels resulting in annovulation, implantation failure or luteal phase deficit (David and Watts 1989). Copper has also been shown to block the absorption of many essential minerals directly involved with reproductive pathways, especially zinc. Copper ions are bound to metallothionen with a greater infinity than zinc, so often zinc deficiencies occur in direct titre to copper levels. Zinc deficiency affects various processes involving both male and female reproduction, despite copper toxicity being more common in females (Watts and David, 2003).

In this study there are high levels of serum copper concentration in the women with (PCOS) in comparison with the fertile women. The PCOS patients also had a statistically and significantly higher amount of copper than the control group (Kurdoglu *et al.*, 2012). The survey conducted by Celik on copper, homocysteine, and early vascular disease in lean women

with PCOS showed high copper levels in PCOS patients (Celik *et al.*, 2013). A similar result was obtained by a study on mice (Chen *et al.*, 2015). Previous study has shown that patients with PCOS copper is involved in the metabolism of oxygen and plays an important role in free radical reactions (Chasapis *et al.*, 2012). Moreover, a significant higher copper level in women with PCOS was reported by (Kurdoglu *et al.*, 2012 ; Chakraborty *et al.*, 2013). Physio-chemically, copper is considered as prooxidant and oxidant metal (Klotz *et al.*, 2003). A low copper level can lead to decreased activity of superoxide dismutase and sequentially increases superoxide radical generations and increases oxidative stress (Hawk *et al.*, 2003). On the other hand, a high copper level can lead to generation of reactive oxygen.

Copper is one of the trace elements in the human body. In this study, the copper content in PCOS patients was higher than that in fertile women group; this finding is consistent with that of previous studies. Chronic copper overload can result in oxidative damages and is associated with PCOS patients (Kurdoglu *et al.*, 2012). High copper concentrations can contribute to the release of LH and adrenocorticotrophic hormone by affecting the pituitary gland, which affects ovulation. Previous research has shown that copper and zinc may cause oxidative stress to act on PCOS by affecting the hormone levels (Zheng *et al.*, 2015).

Table (3 – 6):- Serum iron and copper levels of control and infertile groups

Groups	NO.	Fe ($\mu g/dl$)	Cu ($\mu g/dl$)
		<i>mean $\pm$ SD</i>	<i>mean $\pm$ SD</i>
Control	34	81.0 $\pm$ 19.2 ^c	102.0 $\pm$ 25.1 ^c
1^o UI	34	122.0 $\pm$ 29.2 ^a	181.5 $\pm$ 59.0 ^a
2^o UI	34	112.1 $\pm$ 29.7 ^b	171.1 $\pm$ 47.4 ^a
1^o PCO	34	81.8 $\pm$ 14.5 ^c	118.41 $\pm$ 24.5 ^b
2^o PCO	34	82.1 $\pm$ 15.1 ^c	116.2 $\pm$ 23.5 ^b

- Legend as in table (3.2)

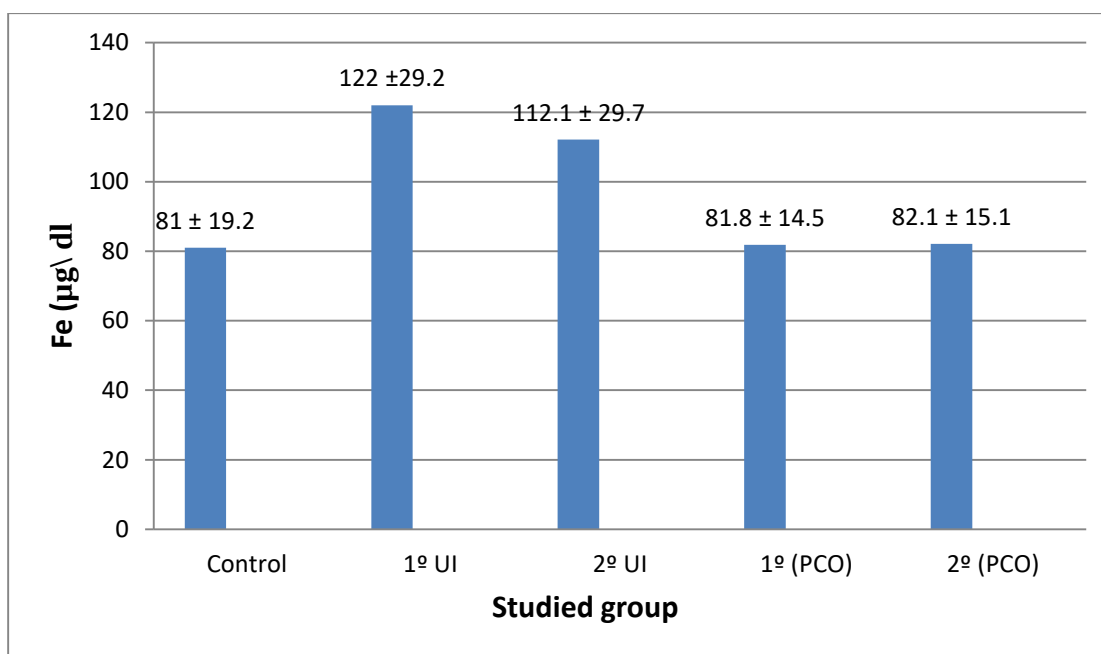


Figure (3 – 14):- Serum iron levels of control and infertile women groups

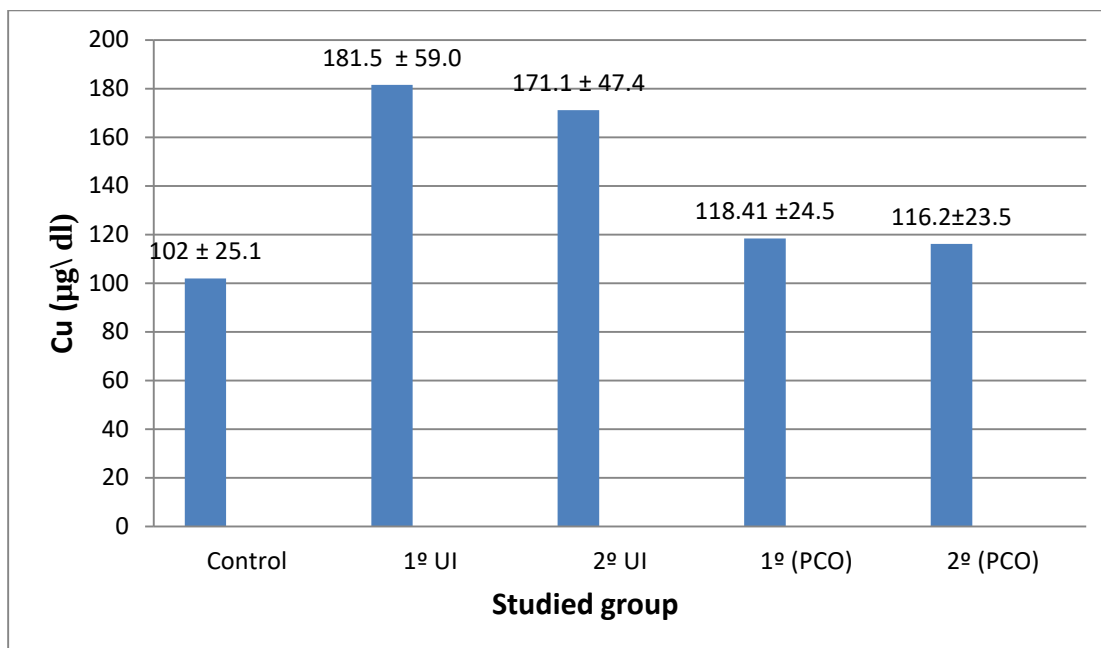


Figure (3 – 15):- Serum copper levels of control and infertile women groups

3.3. Molecular Study for Glutathione Disulfide Reductase Gene (GSR):

3.3.1. DNA Extraction and Polymerase Chain Reaction

Technique PCR:

The DNA was extracted from human women blood (1° UI: Primary unexplained infertility (UI), 2° UI: Secondary unexplained infertility 1°PCO: Primary explained infertility (PCOS) and 2° PCO: Secondary explained infertility (PCOS), using the commercial kit methods, the DNA extraction results were accepted, concentration and purity were determined using Nanodrop 1000 spectrophotometer at 260/280nm and figure (3-16). DNA concentration ranged between 135.5-637.7ng/µl and purity ranging from 1.99 to 2.2, as well as visually by horizontal gel electrophoresis in 0.8% agarose figure (3-17).

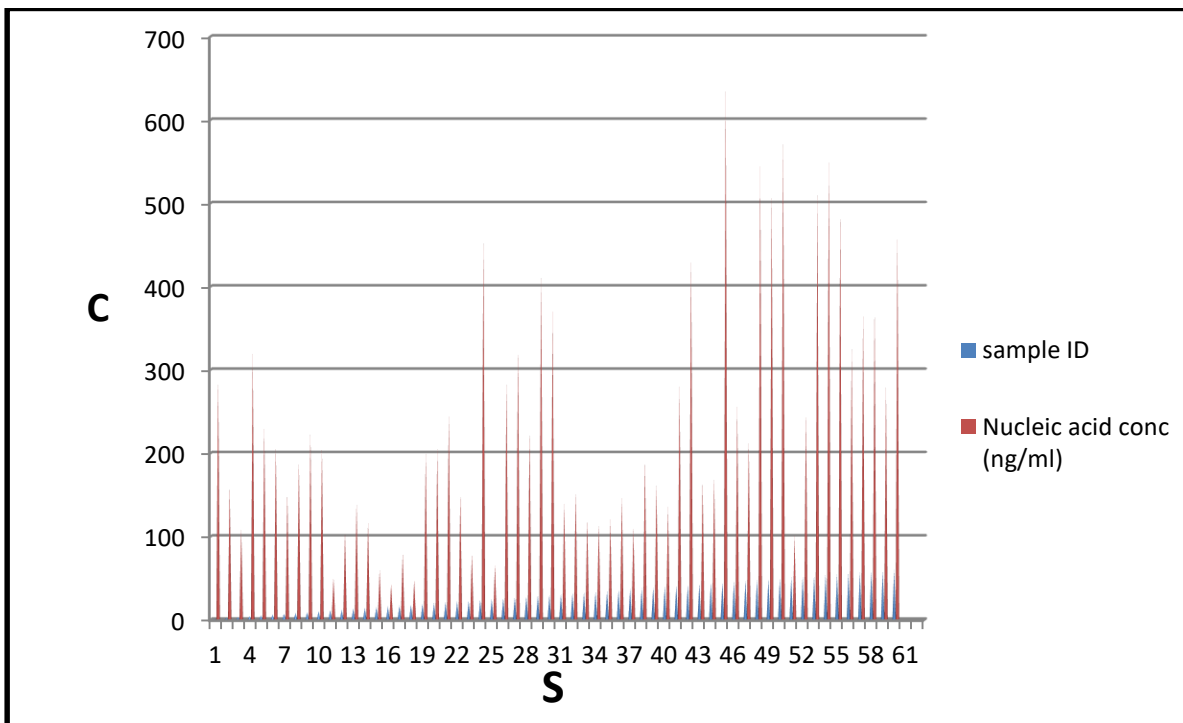


Figure (3-16): Concentration of genomic DNA (ng/ul) extracted from human womem blood (1° UI, 2° UI, 1° PCO and 2° PCO), using the commercial kit methods. C: concentration, S: samples.

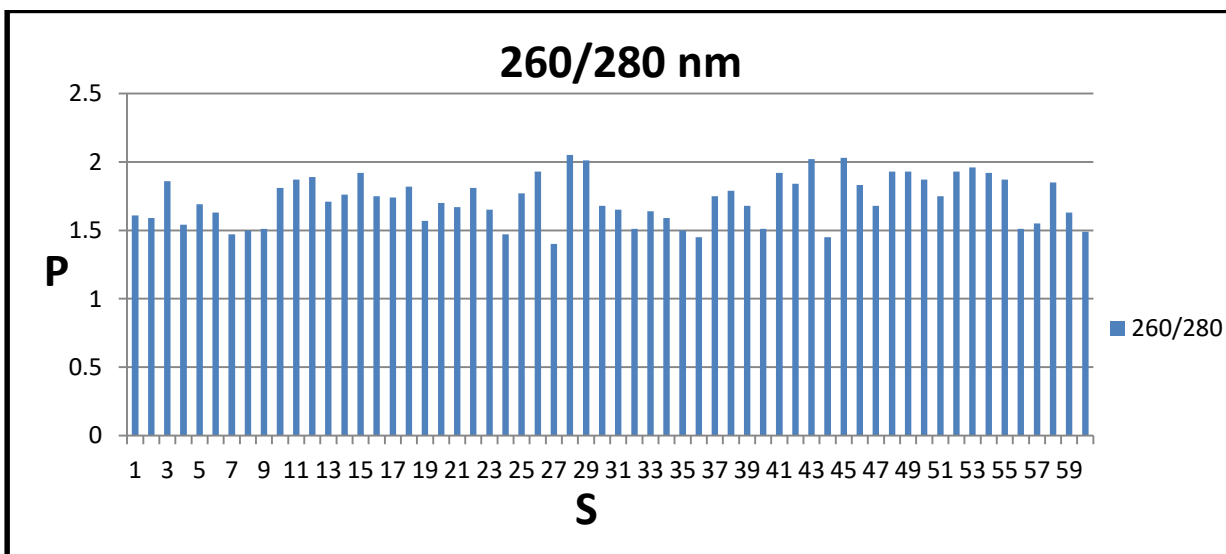


Figure (3-17): Purity of genomic DNA (260/280) extracted from human womem blood (1°UI, 2°UI, 1°PCO and 2° PCO), using the commercial kit methods. P: purity, S: samples

After the completion of the extraction process shows the DNA bundles extracted from human women blood and good yield of genomic DNA as show in figure (3-18).

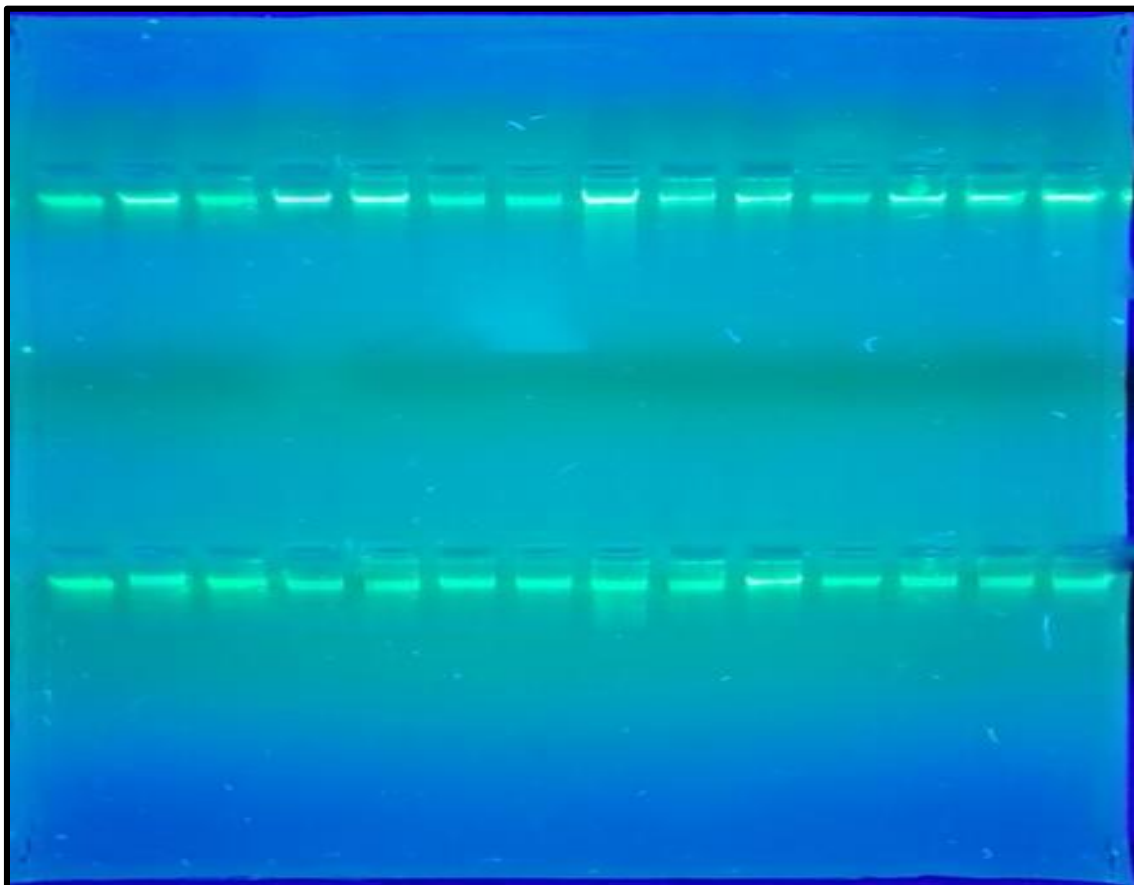


Figure (3-18): Gel electrophoresis of genomic DNA extraction from blood 0.8% agarose gel at 5 volt/cm for 1:15 min. then visualized under U.V after staining with Red safe Nucleic acid staining solution.

The Kit provides a simple and convenient technique to isolate high quality DNA from fungi; this kit features fast, easy processing, produced high quality and purity of DNA. This highly versatile reagent system is able to extract genomic DNA from various other samples, such as cultured bacteria. Purified DNA is suitable for DNA archiving, PCR, AFLP, RFLP/RAPD Southern Blotting, Real-time PCR. Abdulateef (2014) and Rassin (2015)

used commercial kit for DNA extraction from blood and tissue, the extracted DNA used successfully for the specific PCR and Random Amplification of Polymorphic DNA RAPD PCR analysis. Molecular biological studies of blood, such as the PCR techniques, require pure DNA (Ahmadikhah, 2009). The isolation of DNA from blood sample was faster compared with the isolation of DNA by cetyltrimethylammonium bromide CTAB method (Al-Hadeithi, 2012). Most laboratories prefer a simple and fast procedure for obtaining blood, cell line and animal tissue genomic DNA for PCR technique (Ahmadikhah, 2009). The quality and quantity were sufficient for polymerase chain reaction amplification and Southern blot analysis (Saitoh *et al.*, 2006). Commercial kit DNA extraction procedure for isolation of high-quality DNA were used in this study.

3.3.2. Detection for Glutathione Disulfide Reductase (GSR) Gene in the Studied Group:

The results obtained from polymerase Chain Reaction (PCR) was done for the detection of (GSR) gene in samples DNA extracted from women classified as (1° UI, 2° UI, 1° PCO and 2° PCO). The amplification of the (GSR) gene gave positive results for all the subjected samples figure (3-19), also the amplification revealed Polymorphism product of two molecular weight 250 bp. PCR negative control was carried out in each experiment to determine any contamination and due to careful employment of laboratory techniques all negative controls appeared as empty gel lanes through all experiments of optimization. Each reaction was repeated twice and only reproducible bands were considered for analysis (McPherson and MØller, 2001; Sambrook, and Russell 2001 ; Roux, 2009).

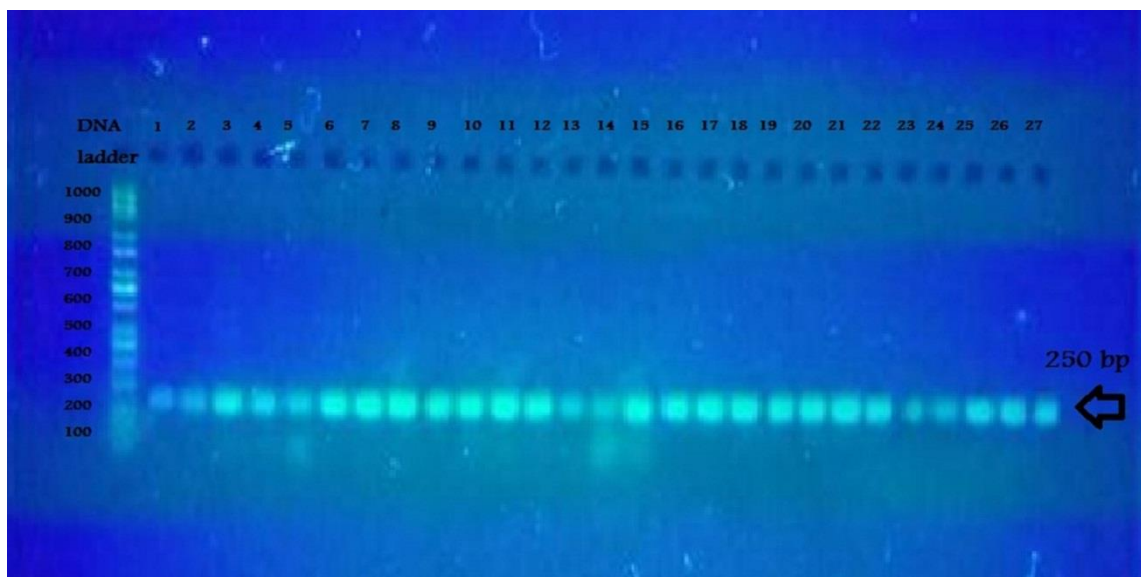


Figure (3-19): Agarose gel electrophoresis of PCR amplification products of (GSR) in the samples of (1°UI, 2°UI, 1°PCO, 2°PCO). 2% agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular weight marker (10000 bp ladder). Positive amplification of 250 bp for (GSR) gene. Lane (1-5): 1° UI, Lane (6-10): 2° UI, Lane (11-15): 1° PCO and Lane (16-20): 2° PCO, Lane (21-27): control

3.3.3: Purification the Samples from Gel:

After DNA extraction from samples then purification and concentration measurement then diagnosis by polymerase chain reaction (PCR) and appeared the results must be send the results to purpose sequencing processing but before that must be purification from the gel after PCR. After electrophoresis to products the PCR then staining and visualized under U.V light when the band appeared taken by cut its and followed the Gel extraction method, after extraction bands from gel work measurement to purification and concentration the product by Nanodrop after extraction processing send samples to work sequence analysis (Ramalho-Santos *et al.*, 2013).

3.3.4 Sequencing and Alignment of NCBI:

The results shown in Figure (3-20) indicated that a yield of single band of the desired product of (GSR). Region gene of (1° UI, 2° UI, 1° PCO and 2° PCO), was obtained from samples related to molecular weight 250 bp. PCR product samples were sent for sequence analysis; PCR product and 25 µl (10 pmol) from the forward primer. The samples were treated with AB13730XL APPLIED BIOSYSTEMS machine in national instrumentation center for environmental management NICM/USA company online at (http://nicem.snu.ac.kr/main/?en_skin=index.html).

The result of the sequence analysis was analysed by blast in the National Center Biotechnology Information (NCBI) online at (<http://www.ncbi.nlm.nih.gov>) and BioEdit program to detect polymorphism in (GSR) in the 1° UI group, found 4 mutations insertion (refers to the insertion of T nucleotide base pairs) showed 99% compatibility and score (217) bits(117) and expect 6e-53 as shown in figure (3-20), with the wild type of (GSR)gene from Gene Bank as shown in figure (3-20) and table (3-7). 2° UI, found 8 mutations between 5 insertion (refers to the insertion of T nucleotide base pairs) showed 99% compatibility and score (217) bits(117) and expect 6e-53 and 3 transversion (refers to the substitution of a Thiamine to adenine (T ↔ A) showed 98% compatibility score (219) bits (118) and expect 2e-53 as shown in Figure (3-20), with the wild type of (GSR)gene from Gene Bank as shown in Figure (3-20) and table (3-7), at the (1° PCO and 2° PCO) groups, found 9 mutations between 5 insertion (refers to the insertion of T nucleotide base pairs) showed 99% compatibility and score (228) bits (123) and expect 3e-56 and 4 transversion (refers to the substitution of a Thiamine to adenine (T ↔ A) showed 98% compatibility

score (219) bits (118) and expect 2e-53 at the (1° PCO and 2° PCO), as shown in Figure (3-20), with the wild type of (GSR) gene from Gene Bank as shown in Figure (3-20) and table (3-7).

The expectation value is defined to give an estimate of the number of times expected to get the same similarity coincidental and the lower the value of E. This indicates that the degree of similarity was high between sequences which give greater confidence. The value of a very close to zero means that these sequences are identical and the bit Score: statistical measure of the moral similarity and the higher value indicates that the high degree of similarity, and if dropped from the class of 50 points, the sense that there is no similarity mention based on (GSR) Gene sequences. The new gene (Homo sapiens glutathione-disulfide reductase (GSR) gene) has been added partial cds GenBank: (MF576503.1), as shown in appendix (1)

Homo sapiens glutathione-disulfide reductase (GSR), RefSeqGene on chromosome 8
Sequence ID: [NG_027719.1](#) Length: 56909 Number of Matches: 1
Related Information
[Map Viewer](#)-aligned genomic context
Range 1: 52043 to 52166 [GenBankGraphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
219 bits(118)	2e-53	123/125(98%)	1/125(0%)	Plus/Plus
Query 4	GTCTGTGCAAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAAACATAATAGTGCCTTGC	63		
	■			
Sbjct 52043	GTCTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAAACATAATAGTGCCTTGC	52102		
Query 64	AAAATGGACAGGGATGGCAGTATTTTGCAGGGGTGGAAAGGAGGGAGACCAAGTCAGTTG	123		
	■			
Sbjct 52103	AAAATGGACAGGGATGGCAGTATTTTGCAGGGGTGGAAAGGAGGGAGACCAAG-CAGTTG	52161		
Query 124	AGCCT 128			
Sbjct 52162	AGCCT 52166			

CHAPTER THREE Results & Discussion

Homo sapiens glutathione-disulfide reductase (GSR), RefSeqGene on chromosome 8

Sequence ID: [NG_027719.1](#) Length: 56909 Number of Matches: 1

Related Information

[Map Viewer](#)-aligned genomic context

Range 1: 52039 to 52164 [GenBankGraphics](#) Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
228 bits(123)	3e-56	126/127(99%)	1/127(0%)	Plus/Plus
Query 1	AATGGTCTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAAACATAAATAGTGCC	60		
Sbjct 52039	AATGGTCTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAAACATAAATAGTGCC			
52098				
Query 61	TTGCAAAATGGACAGGGATGGCAGTATTTTCAGGGGTGGAAAGGAGGGAGACCAAGTCA	120		
Sbjct 52099	TTGCAAAATGGACAGGGATGGCAGTATTTTCAGGGGTGGAAAGGAGGGAGACCAAG-CA			
52157				
Query 121	GTTGAGC 127			
Sbjct 52158	GTTGAGC 52164			

Homo sapiens glutathione-disulfide reductase (GSR), RefSeqGene on chromosome 8

Sequence ID: [NG_027719.1](#) Length: 56909 Number of Matches: 1

[Map Viewer](#)-aligned genomic context

Range 1: 52046 to 52166 [GenBankGraphics](#) Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
219 bits(118)	2e-53	121/122(99%)	1/122(0%)	Plus/Plus
Query 1	TGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAAACATAAATAGTGCCTTGCAAA	60		
Sbjct 52046	TGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAAACATAAATAGTGCCTTGCAAA			
52105				
Query 61	ATGGACAGGGATGGCAGTATTTTCAGGGGTGGAAAGGAGGGAGACCAAGTCAGTTGAGC	120		
Sbjct 52106	ATGGACAGGGATGGCAGTATTTTCAGGGGTGGAAAGGAGGGAGACCAAG-CAGTTGAGC			
52164				
Query 121	CT 122			
Sbjct 52165	CT 52166			

Range 1: 52045 to 52164 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
217 bits(117)	6e-53	120/121(99%)	1/121(0%)	Plus/Plus
Query 1	CTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAA	CTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAA	CTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAA	60
Sbjct 52045	CTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAA	CTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAA	CTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAA	52104
Query 61	AATGGACAGGGATGGCAGTATTTTGCAGGGGTGGAAAGGAGGGAGACCAAGTCAGTTGAG	AATGGACAGGGATGGCAGTATTTTGCAGGGGTGGAAAGGAGGGAGACCAAGTCAGTTGAG	AATGGACAGGGATGGCAGTATTTTGCAGGGGTGGAAAGGAGGGAGACCAAGTCAGTTGAG	120
Sbjct 52105	AATGGACAGGGATGGCAGTATTTTGCAGGGGTGGAAAGGAGGGAGACCAAG-CAGTTGAG	AATGGACAGGGATGGCAGTATTTTGCAGGGGTGGAAAGGAGGGAGACCAAG-CAGTTGAG	AATGGACAGGGATGGCAGTATTTTGCAGGGGTGGAAAGGAGGGAGACCAAG-CAGTTGAG	52163
Query 121	C 121	C 121	C 121	
Sbjct 52164	C 52164	C 52164	C 52164	

Figure (3-20): Sequencing of sense flanking the partial (GSR) gene of (1° UI, 2° UI, 1° PCO and 2° PCO), compared with standard GSR, and obtained from Gene Bank. Query represents of sample; Sbjct represent of database of National Centre Biotechnology Information (NCBI).

Table (3-7): Represent type of polymorphism in (GSR) gene of control and infertile groups

No. Of sample	Type of substitution	Location	Nucleotide	Range of nucleotide	Sequence ID	Score	Expect	Identities	SOURCE
11 (1°UI)	INSERTION	52155	▼ T	52045 to 52164	ID: NG_027719.1	117	6e-53	99%	Homo sapiens glutathione-disulfide reductase (GSR)
12 (1°UI)	INSERTION	52155	▼ T	52046 to 52166	ID: NG_027719.1	118	2e-53	99%	Homo sapiens glutathione-disulfide reductase (GSR)
13 (1°UI)	INSERTION	52155	▼ T	52048 to 52166	ID: NG_027719.1	116	2e-52	99%	Homo sapiens glutathione-disulfide reductase (GSR)
15 (1°UI)	INSERTION	52155	▼ T	52045 to 52165	ID: NG_027719.1	118	2e-53	99%	Homo sapiens glutathione-disulfide reductase (GSR)
22 (2°UI)	Transversion	52051	T>A	52047 to 52166	ID: NG_027719.1	114	3e-51	98%	Homo sapiens glutathione-disulfide reductase (GSR)
	INSERTION	52155	▼ T						
23 (2°UI)	INSERTION	52155	▼ T	52045 to 52164	ID: NG_027719.1	117	6e-53	99%	Homo sapiens glutathione-disulfide reductase (GSR)
24 (2°UI)	Transversion	52051	T>A	52043 to 52166	ID: NG_027719.1	118	2e-53	98%	Homo sapiens glutathione-disulfide reductase (GSR)
	INSERTION	52155	▼ T						
25 (2°UI)	Transversion	52051	T>A	52042 to 52166	ID: NG_027719.1	119	4e-54	98%	Homo sapiens glutathione-disulfide reductase (GSR)
	INSERTION	52155	▼ T						
27 (2°UI)	INSERTION	52155	▼ T	52041 to 52166	ID: NG_027719.1	123	3e-56	99%	Homo sapiens glutathione-disulfide reductase (GSR)
2 (1° pcos)	INSERTION	52155	▼ T	52039 to 52164	ID: NG_027719.1	123	3e-56	99%	Homo sapiens glutathione-disulfide reductase (GSR)
3 (1° pcos)	INSERTION	52155	▼ T	52042 to 52164	ID: NG_027719.1	120	1e-54	99%	Homo sapiens glutathione-disulfide reductase (GSR)

CHAPTER THREE Results & Discussion

4 (1° pcos)	Transversion	52051	T>A	52043 to 52164	ID: NG_027719.1	116	2e-52	98%	Homo sapiens glutathione-disulfide reductase (GSR)
	INSERTION	52155	▼ T						
5 (1° pcos)	Transversion	52051	T>A	52045 to 52166	ID: NG_027719.1	116	2e-52	98%	Homo sapiens glutathione-disulfide reductase (GSR)
	INSERTION	52155	▼ T						
10(1° pcos)	INSERTION	52155	▼ T	52046 to 52166	ID: NG_027719.1	118	2e-53	99%	Homo sapiens glutathione-disulfide reductase (GSR)
1 (2° pcos)	INSERTION	52155	▼ T	52039 to 52164	ID: NG_027719.1	123	3e-56	99%	Homo sapiens glutathione-disulfide reductase (GSR)
2 (2° pcos)	INSERTION	52155	▼ T	52042 to 52164	ID: NG_027719.1	120	1e-54	99%	Homo sapiens glutathione-disulfide reductase (GSR)
3(2° pcos)	Transversion	52051	T>A	52043 to 52164	ID: NG_027719.1	116	2e-52	98%	Homo sapiens glutathione-disulfide reductase (GSR)
	INSERTION	52155	▼ T						
4 (2° pcos)	Transversion	52051	T>A	52045 to 52166	ID: NG_027719.1	116	2e-52	98%	Homo sapiens glutathione-disulfide reductase (GSR)
10(2° pcos)	INSERTION	52155	▼ T	52046 to 52166	ID: NG_027719.1	118	2e-53	99%	Homo sapiens glutathione-disulfide reductase (GSR)
33 Control	INSERTION	52155	▼ T	52042 to 52164	ID: NG_027719.1	120	1e-54	99%	Homo sapiens glutathione-disulfide reductase (GSR)
34 Control	INSERTION	52155	▼ T	52041 to 52165	ID: NG_027719.1	122	1e-55	99%	Homo sapiens glutathione-disulfide reductase (GSR)
35 Control	INSERTION	52155	▼ T	52041 to 52166	ID: NG_027719.1	123	3e-56	99%	Homo sapiens glutathione-disulfide reductase (GSR)
36 Control	INSERTION	52155	▼ T	52059 to 52166	ID: NG_027719.1	105	3e-46	99%	Homo sapiens glutathione-disulfide reductase (GSR)
37 Control	INSERTION	52155	▼ T	52042 to 52166	ID: NG_027719.1	122	1e-55	99%	Homo sapiens glutathione-disulfide reductase (GSR)

This study was undertaken to investigate genetic variations in the genes of GSR in women with unexplained infertility compared to fertile controls. Molecular understanding of the role of glutathione disulfide reductase (GSR) gene factor mutation and polymorphism may be effects function in reproduction, where genetic reasons may be suspected. The present results may be related to glutathione disulfide reductase (GSR) protein is transcribed from a single gene copy and between species. There is a high degree of sequence similarity.

We can understand the current results related to different groups from suggestion display in animal previous results studies where the homozygous GSR gene was inactivated in female transgenic mice, showed that implantation of the blastocyst in the uterine failed (Zorrilla *et al.*, 2013). When the blastocyst was transferred from the GSR deficient mice to normal mice the implantation succeeded. Infusion of GSR into the uterine lumen of the transgenic mice also resulted in implantation. Furthermore, high levels of GSR mRNA have been found in the glandular epithelium of mouse uterus during implantation (Sousa *et al.*, 2011). And GSR treatment of mice leading to increased GSR mRNA values in the uterus promotes embryo implantation (Santos *et al.*, 2006).

The results obtained from sequencing may be attributed to the high sequence identity among (GSR) repeat regions in our study group. It is anticipated that the reductive stress will interfere with the process of protamination and DNA synthesis. Both processes imply the resolution of the so-called supercoiling of DNA (Van Blerkom *et al.*, 2011), which happens by extensive cut and paste work by the enzymes topoisomerase among (GSR) that's may be generates mutation breaks to allow distension of the unexplained infertility (Jansen *et al.*, 2014).

Thereafter molecular analysis of unexplained infertility allows understanding the process of consumes huge amounts of oxidized NAD (NAD⁺) as the ribose source for the synthesis of poly (ADP-ribose) PAR: as DNA replication starts there is a sharp fall in cell (GSR) content and activation of ATP hydrolysis to feed NAD⁺ availability (GSR) (Jansen *et al.*, 2004; Jansen *et al.*, 2014). Any reductive stress may cause imbalance which may lead to unexplained infertility of NAD toward its reduced form (NADH) resulting in shortage of NAD⁺, in metabolic inhibition of (GSR) and in accumulation of unrepaired double strand breaks for imbalanced mutation activity. (Van Blerkom *et al.*, 2011).

Another problem is that, whatever the composition of antioxidant cocktails, it will have to contain a finite number of substances. Each of these substances will influence a finite number of oxy-redox reactions that, according to the dose of antioxidants administered, will be possibly imbalanced toward the reductive stress. Meantime, all the other oxyredox reactions will remain unaffected. The final outcome is failure in equilibrating the intracellular environment and a possible reductive stress in selected pathways. (Chinnery *et al.*, 2005; Goswami, 2005; Zorrilla *et al.*, 2013). This approach may be clinically advantageous any time the objective is the correction of a specific, well-known pathway but it is not suitable if the aim is to achieve a perfect oxy-redox balance and efficient DNA replication and protamination as required by gametogenesis (Reeve *et al.*, 2012).

Human GSR is encoded by a single gene (*GSR*, GeneID: 2936, MIM 138300), located on chromosome 8p21.1 and consisting of 13 exons spanning 50 kb (Van Blerkom *et al.*, 2011). The gene contains 2 in-frame

start codons that are thought to initiate the synthesis of mitochondrial and cytosolic GR (Jansen *et al.*, 2004; Jansen and Pallardó 2014)

3.3.5. Genotyping and Allele Frequency of (GSR) Gene:

In this study, genotype and allele frequency in studied groups of glutathione disulfide reductase (GSR) gene and its possible correlation to studied groups (1° UI, 2° UI, 1° PCO and 2° PCO) risk was investigated among the Iraqi women. The polymorphisms of the TT and TA in (GSR) genotype in H-W observed frequency showed highly significant different ($p < 0.05$) in the patients compared to the control group as summarized in Table (3.8). The allele frequency in UI patients and control groups show polymorphisms in TT in UI patients showed highly significant different $P < 0.05$, with H-W observed frequency percentage 100.0 % while the percentage in patients UI show H-W observed frequency percentage 69.12%, OR (0.03) with CI 95% reach to (0.01-0.51). Also the results showed the polymorphisms in TA in UI patients $P < 0.05$ with H-W observed frequency percentage 100.0 % while the percentage in UI group show H-W observed frequency percentage 30.88%, OR 31.23 with CI 95% reach to (1.90-512.43) as show in (appendix2) .

The genotype and allele frequency in 1° UI patients and control groups show polymorphisms in TT in both patients and control group $P < 0.05$ (appendix 3). Also the polymorphisms of the TA in (GSR) genotype in H-W observed frequency showed highly significant different ($p < 0.05$) in the patients compared to the control group with H-W observed frequency percentage 100.0 % while the percentage in patients 2° UI show H-W observed frequency percentage 61.76%, OR 109.89 with CI 95% reach to (2.66-1866.2), as show in (appendix4).

the polymorphisms of the TA in (GSR) genotype in H-W observed frequency showed highly significant different ($p < 0.05$) in the 2°UI group compared to the 1°UI group with H-W observed frequency percentage in patients 2°UI show H-W observed frequency percentage 61.76% with CI 95% reach to (2.66-1866.2), OR (109.89) as show in (appendix 5). The allele frequency TA polymorphisms in GSR gene in 2° UI group may be associated with an increased risk of secondary unexplained infertility.

The results showed the polymorphisms of the TA in (GSR) genotype in H-W observed frequency showed highly significant different ($p < 0.05$) in the PCOS patients compared to the control group with the percentage in (PCOS) patients 41.18%, OR 48.56 with CI 95% reach to (2.97-792.49) based on HWE as show in (appendix 6). Also the polymorphisms of the TA in (GSR) genotype in H-W observed frequency showed highly significant different ($p < 0.05$) in the (1°PCO) patients compared to the control group show H-W observed frequency percentage 41%, OR 48.80 with CI 95% reach to (2.88-827.32) as show in (appendix 8). This results similar the results in (appendix 9). The study showed no significant different ($p > 0.05$) in the genotype and allele frequency of glutathione disulfide reductase (GSR) gene between 1°PCOS and 2°PCOS groups.

The allele frequency TA polymorphisms in this gene in (1°PCOS and 2°PCOS) groups may be associated with an increased risk of primary and secondary explained infertility (PCOS).

Table (3-8): The genotype and allele frequency in control and studied groups

Polymorphisms	Groups								P-value	OR	CI 95%	EF	PF
	Control N=34				Patients N=136								
	H-W observed frequency	%	H-W expected frequency	%	H-W observed frequency	%	H-W expected frequency	%					
TT	34	100.0	34	100.0	87	63.97	91.41	67.22	2.9x10 ⁻⁶	0.05	0.01-0.38	-	0.92
TA	0	0.0	0	0.0	49	36.03	40.17	29.54	2.9x10 ⁻⁶	39.03	2.44-625.11	0.35	
AA	0	0.0	0	0.0	0	0.0	4.41	3.25	-	-	-	0.0	-
Total	34	100	34	100	136	100	136	100					
HWE	P < 0.05				P < 0.05								
Allele percentage frequency													
T	100.0				81.99								
A	0.0				18.01								

HWE: Hardy-Weinberg equilibrium, p: probability, OR: Odd ratio, EF: effective fraction, PF: protective fraction, CI 95%: 95% Confidence interval

3.3.6. Polymorphisms effect on MDA leveles in studied groups.

In this study, the genetic polymorphisms of Glutathione disulfide reductase (GSR) gene and its possible correlation to studied groups (1° UI, 2° UI, 1° PCO and 2° PCO) risk was investigated among the Iraqi women. The distribution of the (GSR) genotype in this study showed highly significant different ($p < 0.01$) in the patients compared to the control group as summarized in Table (3-9).

The effect of MDA in polymorphisms and the distribution of genotype and allele frequency at the women studied groups (1° UI, 2° UI, 1° PCO and 2° PCO) presented in table (3-9), while the effect PRL, TSH, LH, GLH, Cp, SOD, TC, TG, VLDL, LDL, Fe, Cu, in the allele frequency and (GSR) genotyping polymorphisms effect on studied groups summarized in the Appendix (10-23).

Glutathione disulfide reductase (GSR) gene presented. As related with TT genotypes, there were significant differences between control (2.0 ± 0.4) and secondary unexplained infertility group 2°UI ($p < 0.001$), while, TA genotype frequency was significantly higher in secondary unexplained infertility group 2° UI (3.7 ± 1.1) ($p < 0.001$), than in control group (0.0).

As related with AA genotype there were no significant differences between (2° UI) and control group (0.0).

As related with TT (2.0 ± 0.4) genotype, there are significant differences between control group and Primary PCOS group (3.5 ± 1.1), ($p < 0.001$), while, TA genotype frequency was non significantly higher in Primary PCOS group than apparently healthy control group. In contrast, AA genotype frequency was not significantly difference ($p < 0.05$) in control group and

primary PCOS group. Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

The effect of MDA in allele frequency at the TT genotype frequency compare with control group was significantly ($p<0.01$) higher in Primary UI group (3.1 ± 0.3) than in control group (2.0 ± 0.4). TA genotype frequency was significantly ($p<0.01$) higher in Primary UI (2.8 ± 0.8) than in control group. The frequencies of AA alleles were (0.0) in Primary UI and control group, respectively.

Table (3-9) summarizes the frequency distribution genotypes for TT (31.2 ± 0.3) in secondary UI and TT in control (0.0) according to MDA was significantly ($p<0.01$) higher in Secondary UI.

The distribution of the TT genotypes among women in this study was group was significantly ($p<0.01$) higher (3.5 ± 1) in primary PCOS compare with primary UI (3.1 ± 0.3) alleles respectively as shown in Table (3-8). The distribution of TT based on MDA genotypes in secondary PCOS and secondary UI were (3.9 ± 1.1), (3.1 ± 0.3) respectively with significantly high difference ($p<0.05$).

TA genotype frequency was significantly higher in secondary PCOS group (3.7 ± 1.1) ($p<0.05$), than in secondary UI group (3.1 ± 0.3). As related with AA genotype there were no significant differences between Secondary PCOS and Secondary UI group (0.0).

The effect of MDA in polymorphisms and the distribution of genotype and allele frequency at the (GSR) gene presented. As related with TT (2.0 ± 0.4) genotype, there are significant differences between Primary PCOS (3.5 ± 1.1) and secondary UI (3.9 ± 1.1) ($p<0.05$), while, TA genotype frequency was significantly higher in secondary UI (3.7 ± 1.1) ($p<0.05$) than

primary PCOS (0.0) group. In contrast, AA genotype frequency was not significantly difference ($p<0.05$) in Primary PCOS (0.0) and secondary UI (0.0) group. Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2,

Previous study suggest that genetic polymorphisms factors play a major role in the etiology of PCOS (Perez *et al.*, 2007). However, the mode of allele frequency of PCOS strongly correlated with biochemical marker parameters (Amaral *et al.*, 2013). Also a considerable study indicate that PCOS could be a complex trait this means that several genes are interacting with environmental factors to provoke the phenotype (Amaral *et al.*, 2007). In contrast, biochemical parameters, including TSH, LH, PRL, TC, TG, VLDL, LDL, Fe, Cu and hyperandrogenemia, seem to be highly heritable parameters, suggesting that some clinical signs, symptoms, or biochemical parameters of PCOS could be transmitted as autosomal dominant (Ruiz-Pesini *et al.* 1998) or X-linked traits.

While studies of polymorphisms and allele frequency are unable to exclude an autosomal or X-linked dominant mode frequency, the polymorphisms of GSR gene in studied groups (1° UI, 2° UI, 1° PCO and 2° PCO) probably more complex, similar to that most informative risk factor for the development of PCOS (Brower *et al.*, 2007). Furthermore, environmental factors alter the clinical and biochemical presentation in those with genetic predisposition GSR gene in to PCOS groups there are apparent problems which make genetic studies of PCOS difficult to perform (Amaral *et al.*, 2007). The polymorphisms and lack of universally acceptable clinical or biochemical diagnostic criteria have been discussed. PCOS is a disorder which primarily affects women of reproductive age and it is therefore difficult to study in more than one generation. The polymorphisms in GSR

gene in the women with primary unexplained infertility, secondary unexplained infertility, primary explained infertility (PCOS) and secondary explained infertility (PCOS), there is no commonly accepted phenotype may be related to large deletion of the long arm of chromosome 11 was seen in some of the PCOS cases (Ruiz-Pesini *et al.*, 1998). However, there is no large cytogenetic study to identify karyotype abnormalities. There are different candidate genes as a cause of polymorphisms in GSR gene in PCOS; such as genes involved in steroid hormone synthesis and action, genes involved in carbohydrate metabolism and fuel homeostasis, genes involved in gonadotropin action and regulation; and genes in the major histocompatibility region, which could account for certain PCOS features(Perez *et al.*,2007).

Different studies have indicated a genetic susceptibility to PCOS. It was shown that polycystic ovaries and hyperandrogenemia are present in 50% of sisters of affected women (Urbanek, 2007; Goodarzi *et al.*, 2011). Therefore genetic analyse of candidate genes have been performed. Both linkage and association study have suggested that polymorphisms in GSR gene in PCOS can be explained by the interaction of a small number of key genes with environmental, particularly nutritional factors (Brower *et al.*, 2009).

In addition, an increase of mRNA abundance in PCOS has been found in corresponding to the genes of aldehyde dehydrogenase-6 and retinol dehydrogenase-2, which both increases the expression of 17 α -hydroxylase, recent studies have found a significant prevalence of GSR mutation, gene encode the 21-hydroxylase enzyme mimic the primary and secondary PCOS phenotype, in the supposed PCOS population mutations in the insulin receptor gene cause severe insulin resistance (the type A syndrome) associated with PCOS (Lim *et al.*, 2015).

A linkage analysis study found association between a marker that is this locus emerges as a strong candidate region deserving of detailed investigation for the identification of the putative polymorphisms in GSR gene finally abnormalities in gonadotropin secretion, particularly LH, are characteristic of PCOS, which promote exploring genes related to the regulation of LH secretion, LH bioactivity, and LH action (Perez *et al.*,2007).

Table (3-8): Polymorphisms effect on the MDA levels in studied groups.

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
MDA	2.0±0.4	0.0	0.0	3.1±0.3	2.8±0.8	0.0	P<0.001		
	Control(Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.0±0.4	0.0	0.0	31.2±0.3	2.9±0.8	0.0	P<0.001		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.0±0.4	0.0	0.0	3.5±1.1	0.0	0.0	P<0.001		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.0±0.4	0.0	0.0	3.9±1.1	3.7±1.1	0.0	P<0.001		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.5±1.1	0.0	0.0	3.9±1.1	3.7±1.1	0.0	P>0.05		
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.1±0.3	2.8±0.8	0.0	3.9±1.1	3.7±1.1	0.0	P>0.05	P>0.05	
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.5±1.1	0.0	0.0	3.1±0.3	2.8±0.8	0.0	P>0.05	P<0.05	
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.9±1.1	3.7±1.1	0.0	3.1±0.3	2.8±0.8	0.0	P<0.05	P<0.05	
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.5±1.1	0.0	0.0	3.9±1.1	3.7±1.1	0.0	P>0.05	P<0.05	
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.9±1.1	3.7±1.1	0.0	3.1±0.3	2.8±0.8	0.0	P<0.05	P<0.05	

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

3.3.7. Correlation Relationships:

The biochemical parameters in the studied groups (1° UI, 2° UI, 1° PCO and 2° PCO) in the present study was studied statistically. Analysis of the Iraqi patients revealed that there was significance difference between the regression factor of control TT genotype with the parameters (P value <0.01), but the regression factor of primary explained infertility (PCOS) and TT genotype based on the parameters as show in appendix (26) . Genotyping frequency correlated with recurrence. In the current study, there was significant association between primary PCO in TA genotype, secondary PCO TT genotype, secondary PCO in TA, primary UI TT genotype, secondary UI in TT genotype, secondary UI in TA genotype with the chemical parameters MDA, Cp, GLH, TSH, Fe, LH, Cu, PRL, TC, TG, VLDL, LDL and SOD. These results are in accordance with other studies that examined these biochemical marker with (1° UI, 2° UI, 1° PCO and 2°PCO) in exclusively reported that increased as show in the Appendix (24-30).

Related to GSR gene in to PCOS groups also is includes a variety of potential signs and symptoms, including biochemical or clinical hyperandrogenism, polycystic ovaries, and hyperinsulinemia, but no single diagnostic criterion is recommended for diagnosis of this disorder. In addition, the allele frequency TT, AT, and AA Polymorphisms in GSR gene PCOS is associated with an increased risk of primary and secondary PCOS, Insulin resistance and elevated serum LH levels are also common features in PCOS (Corte's-Wanstreet *et al.*, 2009).

Among different criteria, hyperandrogenemia, specifically elevated bioavailable testosterone, and the recognition of oligoovulation are

academically appealing. Many support a combination of hyperandrogenemia and oligoovulation, in the absence of known causes, as diagnostic criteria. While, many others are using ovarian morphology to identify and diagnose the syndrome (Devine *et al.*, 2012). These three are the major criteria but in order to make the diagnosis it needs to fulfil only two out of three. This makes PCOS a heterogeneous disease. Normal ovulatory women with PCO (referring only to the ovarian morphology) are not considered having PCOS. A subgroup of these women may, however, have subtle abnormalities resembling PCOS (Noda *et al.*, 2012).

Although endogenous H_2O_2 is an important signaling molecule, high levels of H_2O_2 are toxic to cells. Exogenous H_2O_2 at concentrations (0.5) mM rapidly induces cytotoxicity in cultured COV434 cells, a human granulosa cell tumor line, which displays many characteristics of normal granulosa cells, such as synthesis of estradiol in the presence of androstenedione and FSH (Corte-Wanstreet *et al.*, 2009). Stable overexpression of GSR Gene increases cellular GSH concentrations and protects against H_2O_2 -induced cytotoxicity (Corte-Wanstreet *et al.*, 2009). In primary PCOS cells, H_2O_2 dose-dependently inhibits progesterone synthesis via inhibition of steroidogenic acute regulatory (StAR) protein expression (Yu *et al.*, 2012).

StAR regulates the rate-limiting step in steroid synthesis, the transfer of cholesterol to the inner mitochondrial membrane. Neonatal rodent ovaries are enriched for primordial follicles, making cultured neonatal ovaries an ideal model system in which to study the effects of toxicants on primordial follicles. In contrast to the adverse effects of H_2O_2 on steroidogenically allele frequency TT, AT, and AA Polymorphisms in GSR, primordial and small primary PCOS in cultured neonatal (postnatal day 4) rat ovaries are

relatively resistant to H₂O₂ concentrations below 6 mM applied for 8 days (Devine *et al.*, , 2012). At the other hand biochemical parameters effects on, primordial and primary follicles display morphological signs of atresia (Devine *et al.*, 2012).

Cells possess numerous enzymatic and nonenzymatic antioxidant systems that function to tightly regulate cellular levels of ROS/RNS. Deficiency of the antioxidant has profound effects on female fertility. Expression of many ovarian antioxidant genes is regulated by gonadotropin hormones, suggesting that these antioxidants may play important roles in ovarian function. Numerous studies in recent years have examined the biological effects of the deletion of genes encoding antioxidant enzymes and their cofactors, including the effects on ovarian function (Luderer, 2014).

The eCG-induced increased GSR expression is localized to theca internal cells of growing follicles, and subsequent administration of an ovulatory dose of hCG increases theca cell expression further and upregulates expression in granulosa cells as well (Sasak *et al.*, 1994). Global deletion of Sod1 has been reported by two groups using different gene-targeting strategies (Ho *et al.*, 1998). Both groups reported that female Sod1-null mice were subfertile but reported somewhat different ovarian phenotypes. Matzuk *et al.* (1998) observed fewer preovulatory follicles and corpora luteal in the ovaries of adult Sod1-null females and concluded that GSR deletion adversely affected antral follicle development and ovulation. Ho *et al.* (1998) reported that GSR-null females had normal ovarian histology and ovulated similar numbers of oocytes during a natural estrous cycle but had smaller litters due to postimplantation embryonic mortality.

Another group subsequently reported that female Sod1-null mice have decreased serum progesterone levels upon superovulation and during

pregnancy and have small corpora lutea with increased numbers of apoptotic cells and fewer blood vessels and demonstrated that embryos null for Sod1 developed normally when transplanted to wild-type dams (Noda, 2012), Another group subsequently reported that female Sod1-null mice have decreased serum progesterone levels upon superovulation and during pregnancy and have small corpora lutea with increased numbers of apoptotic cells and fewer blood vessels and demonstrated that embryos null for Sod1 developed normally when transplanted to wild-type dams. Taken together, the results suggest that GSR is required for normal biochemical in the studied patients group.



CONCLUSION AND RECOMMENDATION

Conclusion and Recommendations

Conclusion:

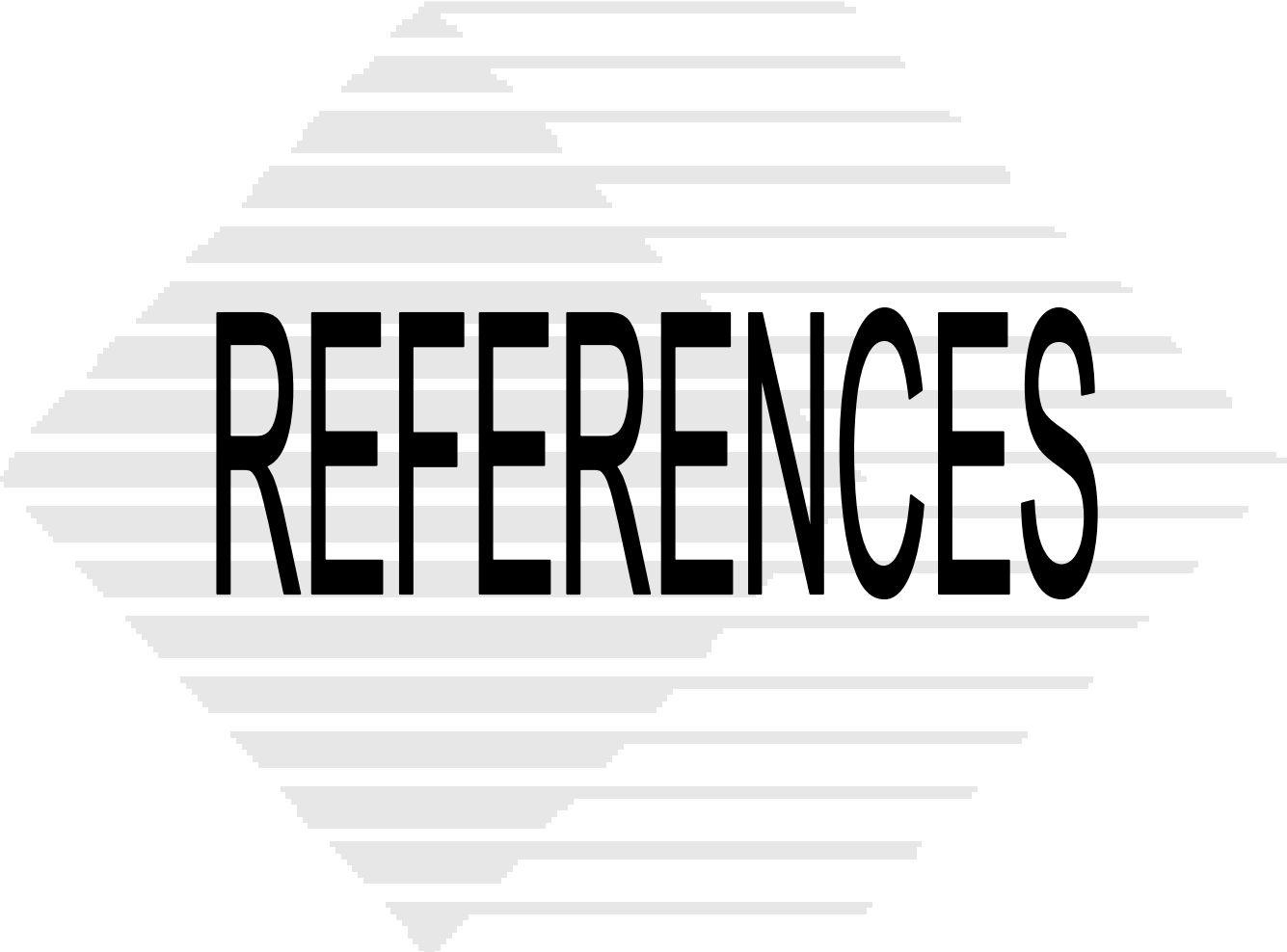
According to the obtained results, this study has reached the following conclusions:

- An alteration in the oxidant –antioxidant status prove the presence of oxidative stress in women with unexplained and (polycystic ovarian syndrome) infertility. This oxidative stress may be cause or result of such infertility.
- The disorder in lipid profile that accompanied infertility may be a cause or result of both two studied types of infertility.
- Copper and Celuoplasmin appear to be associated with the etiopathogenesis of and oxidative stress in unexplained and explained (PCOS) female infertility.
- The present results suggest a relationship between increased iron and copper concentration and unexplained female infertility.
- This study demonstrated the identification of (*GSR gene*) in studied groups and reveals the molecular weight 250bp.
- Sequence analysis was analysed by blast in the National Centre Biotechnology Information (NCBI) detect polymorphism in (GSR) found 3 transversion mutations (T ↔ A) in 2°UI and 4 transversion mutations (T ↔ A) in (1° PCO and 2° PCO) groups.
- The allele frequency TA polymorphisms in GSR gene PCOS are associated with an increased risk of (2°UI, 1° PCO and 2° PCO).
- Biochemical parameters affected by allele frequency and genotyping Polymorphisms of (GSR) and there is a significant correlation between them and each one of studied biochemical parameters.

Conclusion and Recommendations

Recommendations:

- Malondialdehyde level should be carried out a primary test for assessment oxidative stress status in women with unexplained infertility and women with PCOS infertility.
- Assessment of oxidative stress as a cause of unexplained and explained (PCOS) female infertility must be carried out to discriminate oxidative stress infertility from other causes of infertility.
- The study of the role of oxidants in other types of infertility.
- Strategies to overcome oxidative stress in-vitro conditions and balancing between in vivo and in vitro environments can be utilized in assisted reproductive technique, to successfully treat infertility.
- Further studies of PCOS disease are needed to strengthen the role of PRL, TSH, LH and FSH in the development of insulin resistance in similar or, inclusively, different but related clinical conditions.
- The evaluation of other antioxidant for estimating the balance between Oxidative stress marker and antioxidants like catalase enzyme and vitamins (C, A, E, D).
- Supplementation with appropriate antioxidants may be beneficial in reducing the harmful effects of ROS and prevent the future complication in women with unexplained infertility and polycystic ovarian syndrome.
- The study of the role of other trace element such as zinc and selenium in women with unexplained infertility and polycystic ovarian syndrome.
- The detection of polymorphism and mutation of other genes like manganese superoxide dismutase (MnSOD), Insulin receptor substrate (IRS) and Bromodomain-containing protein 2 (Brd2).

A large, light gray diamond shape is centered on the page. It is composed of many thin, horizontal lines of varying lengths that create a sense of depth and movement, resembling a stylized sunburst or a series of overlapping planes.

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APPENDIX

Appendix (1): Homo sapiens glutathione-disulfide reductase (GSR) gene, partial cds

GenBank: [MF576503.1](#)

```

LOCUS       MF576503                126 bp    DNA    linear    PRI 07-
JAN-2018
DEFINITION  Homo sapiens glutathione-disulfide reductase (GSR) gene,
partial
            cds.
ACCESSION   MF576503
VERSION     MF576503.1
KEYWORD     .
SOURCE      Homo sapiens (human)
  ORGANISM   Homo sapiens
              Eukaryota; Metazoa; Chordata; Craniata;
Vertebrata; Euteleostomi;
              Mammalia; Eutheria; Euarchontoglires; Primates;
Haplorrhini;
              Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 126)
  AUTHORS   Khansaa,A.-J.A.
  TITLE     Polymorphism of Glutathione disulfide reductase gene in
Homo
            sapiens
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 126)
  AUTHORS   Khansaa,A.-J.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JUL-2017) Chemistry Department, Thi-Qar
00964, Iraq
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            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                       /product="glutathione-disulfide reductase"
                       /protein_id="AUI41042.1"
                       /translation="MVCANKEEK"

ORIGIN
1  atggtctgtg ctaacaagga agaaaaggta aggaaaaatc cagcaaacta aatagtcct
61  tgcaaaatgg acagggatgg cagtattttg caggggtgga aaggaggag accaagcagt

```

Appendix

121 tgagcc

//

[MF576503.1](#) Homo sapiens glutathione-disulfide reductase (GSR) gene,
partial cds

```
ATGGTCTGTGCTAACAAGGAAGAAAAGGTAAGGAAAAATCCAGCAAATAAATAGTGCCTTG  
CAAAATGG  
ACAGGGATGGCAGTATTTTGCAGGGGTGGAAAGGAGGGAGACCAAGCAGTTGAGCC
```

Appendix

Appendix (2): The genotype and allele frequency in UI patients and control groups

Polymorphisms	Groups								P-value	OR	CI 95%	EF	PF
	Control N=34				UI Patients N=68								
	H-W	%	H-W	%	H-W	%	H-W	%					
	observed frequency		expected frequency		observed frequency		expected frequency						
TT	34	100.0	20	100.0	47	69.12	48.62	71.50	6.6x10 ⁻⁵	0.03	0.01-0.51	-	0.91
TA	0	0.0	0	0.0	21	30.88	17.76	26.11	6.6x10 ⁻⁵	31.23	1.90-512.43	0.30	
AA	0	0.0	0	0.0	0	0.00	1.62	2.38	-	-	-	0.0	-
Total	34	100	20	100	68	100	68	100					
HWE	P < 0.05				P > 0.05								
Allele percentage frequency													
T	100.0				84.56								
A	0.0				15.44								

HWE: Hardy-Weinberg equilibrium, p: probability, OR: Odd ratio, EF: effective fraction, PF: protective fraction, CI 95%: 95% Confidence interval

Appendix (3): The genotype and allele frequency in primary UI patients and control groups.

Polymorphisms	Groups								P-value	OR	CI 95%	EF	PF
	Control N=34				1° UI N=34								
	H-W	%	H-W	%	H-W	%	H-W	%					
	observed frequency		expected frequency		observed frequency		expected frequency						
TT	34	100.0	20	100.0	34	100.0	34.0	100.0	-	-	-	-	-
TA	0	0.0	0	0.0	0	0.0	0.0	0.0	-	-	-	-	-
AA	0	0.0	0	0.0	0	0.0	0.0	0.0	-	-	-	-	-
Total	34	100	20	100	34	100	34	100					
HWE	P < 0.05				P < 0.05								
Allele percentage frequency													
T	100.0				100.0								
A	0.0				0.0								

HWE: Hardy-Weinberg equilibrium, p: probability, OR: Odd ratio, EF: effective fraction, PF: protective fraction, CI 95%: 95% Confidence interval

Appendix

Appendix (4): The genotype and allele frequency in secondary UI patients and control groups

Polymorphisms	Groups								P-value	OR	CI 95%	EF	PF
	Control N=34				2° UI N=34								
	H-W observed frequency	%	H-W expected frequency	%	H-W observed frequency	%	H-W expected frequency	%					
TT	34	100.0	20	100.0	13	38.24	16.24	47.77	9.9x10 ⁻⁹	0.009	0.0-0.15	-	0.95
TA	0	0.0	0	0.0	21	61.76	14.51	42.69	9.9x10 ⁻⁹	109.89	2.66-1866.29	0.61	-
AA	0	0.0	0	0.0	0	0.00	3.24	9.54	-	-	-	0.0	-
Total	34	100	20	100	34	100.0	34	100.0					
HWE	P < 0.05				P < 0.05								
Allele percentage frequency													
T	100.0				69.12								
TA	0.0				30.88								

HWE: Hardy-Weinberg equilibrium, p: probability, OR: Odd ratio, EF: effective fraction, PF: protective fraction, CI 95%: 95% Confidence interval

Appendix (5): The genotype and allele frequency in primary and secondary UI patients

Polymorphisms	Groups								P-value	OR	CI 95%	EF	PF
	1° UI N=34				2° UI N=34								
	H-W observed frequency	%	H-W expected frequency	%	H-W observed frequency	%	H-W expected frequency	%					
TT	34	100.0	34.0	100.0	13	38.24	16.24	47.77	9.9x10 ⁻⁹	0.009	0.0-0.15	-	0.95
TA	0	0.0	0.0	0.0	21	61.76	14.51	42.69	9.9x10 ⁻⁹	109.89	2.66-1866.29	0.61	-
AA	0	0.0	0.0	0.0	0	0.00	3.24	9.54	-	-	-	0.0	-
Total	34	100	34	100	34	100.0	34	100.0					
HWE	P < 0.05				P < 0.05								
Allele percentage frequency													
T	100.0				69.12								
A	0.0				30.88								

HWE: Hardy-Weinberg equilibrium, p: probability, OR: Odd ratio, EF: effective fraction, PF: protective fraction, CI 95%: 95% Confidence interval

Appendix

Appendix (6): The genotype and allele frequency in PCOS patients and control groups

Polymorphisms	Groups								P-value	OR	CI 95%	EF	PF
	Control N=34				PCO N=68								
	H-W observed frequency	%	H-W expected frequenc y	%	H-W observed frequency	%	H-W expected frequency	%					
TT	34	100.0	20	100.0	40	58.82	42.88	63.06	1.1×10 ⁻⁶	0.02	6.27-354.57	0.58	-
TA	0	0.0	0	0.0	28	41.18	22.24	32.70	1.1×10 ⁻⁶	48.56	2.97-792.49	0.41	-
AA	0	0.0	0	0.0	0	0.0	2.88	4.24	-	-	-	0.0	-
Total	34	100	20	100	68	100	68	100					
HWE	P < 0.05				P < 0.05								
Allele percentage frequency													
T	100.0				79.41								
A	0.0				20.59								

HWE: Hardy-Weinberg equilibrium, p: probability, OR: Odd ratio, EF: effective fraction, PF: protective fraction, CI 95%: 95% Confidence interval

Appendix (7): The genotype and allele frequency in primary PCOS patients and control groups

Polymorphisms	Groups								P-value	OR	CI 95%	EF	PF
	Control N=34				1° PCOS N=34								
	H-W observed frequency	%	H-W expected frequenc y	%	H-W observed frequency	%	H-W expected frequency	%					
TT	34	100.0	20	100.0	20	58.82	21.44	63.06	2.3x10 ⁻³	0.02	0.01-0.34	-	0.93
TA	0	0.0	0	0.0	14	41.18	11.12	32.70	2.3x10 ⁻⁵	48.80	2.88-827.32	0.41	-
AA	0	0.0	0	0.0	0	0.0	1.44	4.24	-	-	-	0.0	-
Total	34	100	20	100	34	100	34	100					
HWE	P < 0.05				P > 0.05								
Allele percentage frequency													
T	100.0				79.41								
A	0.0				20.59								

HWE: Hardy-Weinberg equilibrium, p: probability, OR: Odd ratio, EF: effective fraction, PF: protective fraction, CI 95%: 95% Confidence interval

Appendix

Appendix (8): The genotype and allele frequency in secondary PCOS patients and control groups

Polymorphisms	Groups								P-value	OR	CI 95%	EF	PF
	Control N=34				2° PCO N=34								
	H-W	%	H-W	%	H-W	%	H-W	%					
	observed frequency		expected frequency		observed frequency		expected frequency						
TT	34	100.0	20	100.0	19	57.72	20.54	62.03	2.3x10 ⁻³	0.02	0.01-0.32	-	0.92
TA	0	0.0	0	0.0	15	42.28	12.22	33.74	2.3x10 ⁻⁵	48.78	2.86-827.32	0.40	-
AA	0	0.0	0	0.0	0	0.0	1.24	4.23	-	-	-	0.0	-
Total	34	100	20	100	34	100	34	100					
HWE	P < 0.05				P > 0.05								
Allele percentage frequency													
T	100.0				79.41								
A					20.59								

HWE: Hardy-Weinberg equilibrium, *p*: probability, OR: Odd ratio, EF: effective fraction, PF: protective fraction, CI 95%: 95% Confidence interval

Appendix (9): The genotype and allele frequency primary and secondary PCOS patient groups

Polymorphisms	Groups								P-value	OR	CI 95%	EF	PF
	1° PCOS N=34				2° PCO N=34								
	H-W	%	H-W	%	H-W	%	H-W	%					
	observed frequency		expected frequency		observed frequency		expected frequency						
TT	20	58.82	21.44	63.06	20	57.72	20.54	62.03	1.0	1.0	0.39-2.59	0.0	-
TA	14	41.18	11.12	32.70	14	42.28	12.22	33.74	1.0	1.0	0.39-2.59	0.0	-
AA	0	0.0	1.44	4.24	0	0.0	1.24	4.23	-	-	-	0.0	-
Total	34	100	34	100	34	100	34	100					
HWE	P > 0.05				P > 0.05								
Allele percentage frequency													
T	79.41				78.30								
A	20.59				20.41								

HWE: Hardy-Weinberg equilibrium, *p*: probability, OR: Odd ratio, EF: effective fraction, PF: protective fraction, CI 95%: 95% Confidence interval.

Appendix

Appendix (10): Polymorphisms effect on the PRL levels in studied groups.

Groups	Control (Mean± SD)			1° UI (Mean ±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
PRL	14.5±3.9	0	0	14.5±2.9	0	0	P>0.05		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	14.5±3.9	0	0	14.3±16	14.5±3.5	0	P>0.05		
	Control (Mean±SD)			1°PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	14.5±3.9	0	0	16.9±15	15.4±4.2	0	P<0.05		
	Control (Mean±SD)			2°PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	14.5±3.9	0	0	16.5±18	15.5±4.2	0	P<0.05		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	16.9±1.5	15.4±4.2	0	16.5±18	15.5±4.2	0	P>0.05		P>0.05
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	14.5±2.9	0	0	14.3±16	14.5±3.5	0	P>0.05		P>0.05
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	16.9±1.5	15.4±4.2	0	14.5±2.9	0	0	P<0.05		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	16.5±1.8	15.5±4.2	0	14.3±1.6	14.5±3.5	0	P>0.05		P>0.05
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	16.9±1.5	15.4±4.2	0	14.3±1.6	14.5±3.5	0	P<0.05		P>0.05
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	16.5±1.8	15.5±4.2	0	14.5±2.9	0	0	P<0.05		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (11): Polymorphisms effect on the TSH levels in studied groups.

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
TSH	2.3±0.7	0	0	2.3±0.7	0	0	P>0.05		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.3±0.7	0	0	2.2±0.7	2.1±0.6	0	P>0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.3±0.7	0	0	2.9±0.5	2.7±0.9	0	P<0.05		
	Control (Mean± SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.3±0.7	0	0	2.9±0.7	2.8±0.9	0	P<0.05		
	1° PCOS (Mean±SD)			2° PCO (Mean± SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.9±0.5	2.7±0.9	0	2.9±0.7	2.8±0.9	0	P>0.05	P>0.05	
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.3±0.7	0	0	2.2±0.7	2.1±0.6	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.9±0.5	2.7±0.9	0	2.3±0.7	0	0	P<0.05		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.9±0.7	2.8±0.9	0	2.2±0.7	2.1±0.6	0	P<0.05	P<0.05	
	Primary PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.9±0.5	2.7±0.9	0	2.2±0.7	2.1±0.6	0	P<0.05	P<0.05	
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.9±0.7	2.8±0.9	0	2.3±0.7	0	0	P<0.05		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (12): Polymorphisms effect on the LH levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
LH	3.2±0.9	0	0	2.6±0.7	0	0	P>0.05		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.2±0.9	0	0	3.8±1.0	3.6±1.1	0	P>0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.2±0.9	0	0	8.1±1.5	8.1±2.3	0	P<0.001		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.2±0.9	0	0	8.1±1.2	7.9±2.2	0	P<0.001		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	8.1±1.5	8.1±2.3	0	8.1±1.2	7.9±2.2	0	P>0.05		P>0.05
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.6±0.7	0	0	3.8±1.0	3.6±1.1	0	P<0.05		P>0.05
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	8.1±1.5	8.1±2.3	0	2.6±0.7	0	0	P<0.001		P<0.001
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	8.1±1.2	7.9±2.2	0	3.8±1.0	3.6±1.1	0	P<0.001		P<0.001
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	8.1±1.5	8.1±2.3	0	3.8±1.0	3.6±1.1	0	P<0.001		P<0.001
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	8.1±1.2	7.9±2.2	0	2.6±0.7	0	0	P<0.001		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (13): Polymorphisms effect on the FSH levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
FSH	5.4±1.4	0	0	5.3±1.2	0	0	P>0.05		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.4±1.4	0	0	5.4±1.2	5.4±1.5	0	P>0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.4±1.4	0	0	5.5±1.1	5.1±1.5	0	P>0.05		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.4±1.4	0	0	5.6±1.1	5.1±1.5	0	P>0.05		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.5±1.1	5.1±1.5	0	5.6±1.1	5.1±1.5	0	P>0.05		
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.3±1.2	0	0	5.4±1.2	5.4±1.5	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.5±1.1	5.1±1.5	0	5.3±1.2	0	0	P>0.05		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.6±1.1	5.1±1.5	0	5.4±1.2	5.4±1.5	0	P>0.05		
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.5±1.1	5.1±1.5	0	5.4±1.2	5.4±1.5	0	P>0.05		
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.6±1.1	5.1±1.5	0	5.3±1.2	0	0	P>0.05		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (14): Polymorphisms effect on the GSH levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
GSH	524.2±132.2	0	0	305.4±82.5	0	0	P<0.001		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	524.2±132.2	0	0	273.0±73.9	233.2±94.6	0	P<0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	524.2±132.2	0	0	278.2±88.8	275.1±101.7	0	P<0.001		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	524.2±132.2	0	0	280.2±94.1	265.7±81.8	0	P<0.001		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	278.2±88.8	275.1±101.7	0	280.2±94.1	265.7±81.8	0	P>0.05	P>0.05	
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	305.4±82.5	0	0	273.0±73.9	233.2±94.6	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	278.2±88.8	275.1±101.7	0	305.4±82.5	0	0	P>0.05		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	280.2±94.1	265.7±81.8	0	273.0±73.9	233.2±94.6	0	P>0.05	P>0.05	
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	278.2±88.8	275.1±101.7	0	273.0±73.9	233.2±94.6	0	P>0.05	P>0.05	
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	280.2±94.1	265.7±81.8	0	305.4±82.5	0	0	P>0.05		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (15): Polymorphisms effect on the SOD levels in studied groups

Groups	Control (Mean±SD)			Primary UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
SOD	5.4±2.1	0	0	3.0±1.2	0	0	P<0.001		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.4±2.1	0	0	2.8±2.0	3.7±0.5	0	P<0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.4±2.1	0	0	3.0±1.5	3.7±0.6	0	P<0.001		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	5.4±2.1	0	0	3.0±1.4	3.2±0.1	0	P<0.05		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.0±1.5	3.7±0.6	0	3.0±1.4	3.2±0.1	0	P>0.05 P>0.05		
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.0±1.2	0	0	2.8±2.0	3.7±0.5	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.0±1.5	3.7±0.6	0	3.0±1.2	0	0	P>0.05		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.0±1.4	3.2±0.1	0	2.8±2.0	3.7±0.5	0	P>0.05 P>0.05		
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.0±1.5	3.7±0.6	0	2.8±2.0	3.7±0.5	0	P>0.05 P>0.05		
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.0±1.4	3.2±0.1	0	3.0±1.2	0	0	P>0.05		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (16): Polymorphisms effect on the Cp levels in studied groups.

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
Cp	3.7±0.9	0	0	2.9±0.9	0	0	P<0.05		
	Control(Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.7±0.9	0	0	3.3±0.6	2.8±1.0	0	P<0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.7±0.9	0	0	1.5±0.4	1.4±0.5	0	P<0.001		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	3.7±0.9	0	0	1.4±0.4	1.3±0.4	0	P<0.001		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	1.5±0.4	1.4±0.5	0	1.4±0.4	1.3±0.4	0	P>0.05 P>0.05		
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	2.9±0.9	0	0	3.3±0.6	2.8±1.0	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	1.5±0.4	1.4±0.5	0	2.9±0.9	0	0	P<0.001		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	1.4±0.4	1.3±0.4	0	3.3±0.6	2.8±1.0	0	P<0.001 P<0.001		
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	1.5±0.4	1.4±0.5	0	3.3±0.6	2.8±1.0	0	P<0.001 P<0.001		
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	1.4±0.4	1.3±0.4	0	2.9±0.9	0	0	P<0.001		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (17): Polymorphisms effect on the TC levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
TC	139.0±29.2	0	0	146.9±29.3	0	0	P<0.05		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	139.0±29.2	0	0	163.7±14.7	149.1±35.0	0	P<0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	139.0±29.2	0	0	177.4±19.8	151.4±41.8	0	P<0.001		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	139.0±29.2	0	0	175.4±18.4	149.7±41.1	0	P<0.001		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	177.4±19.8	151.4±41.8	0	175.4±18.4	149.7±41.1	0	P>0.05		P>0.05
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	146.9±29.3	0	0	163.7±14.7	149.1±35.0	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	177.4±19.8	151.4±41.8	0	146.9±29.3	0	0	P<0.001		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	175.4±18.4	149.7±41.1	0	163.7±14.7	149.1±35.0	0	P>0.05		P>0.05
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	177.4±19.8	151.4±41.8	0	163.7±14.7	149.1±35.0	0	P>0.05		P>0.05
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	175.4±18.4	149.7±41.1	0	146.9±29.3	0	0	P<0.001		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (18): Polymorphisms effect on the LDL levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
LDL	72.7±21.3	0	0	91.6±23.4	0	0	P<0.05		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	72.7±21.3	0	0	96.0±20.0	88.5±27.8	0	P<0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	72.7±21.3	0	0	106.3±20.1	95.3±27.0	0	P<0.001		
	Control (Mean ±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	72.7±21.3	0	0	106.8±15.2	95.0±25.8	0	P<0.001		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	106.3±20.1	95.3±27.0	0	106.8±15.2	95.0±25.8	0	P>0.05		P>0.05
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	91.6±23.4	0	0	96.0±20.0	88.5±27.8	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	106.3±20.1	95.3±27.0	0	91.6±23.4	0	0	P<0.05		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	106.8±15.2	95.0±25.8	0	96.0±20.0	88.5±27.8	0	P>0.05		P>0.05
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	106.3±20.1	95.3±27.0	0	96.0±20.0	88.5±27.8	0	P>0.05		P>0.05
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	106.8±15.2	95.0±25.8	0	91.6±23.4	0	0	P<0.05		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (19): Polymorphisms effect on the HDL levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
HDL	49.0±14.6	0	0	34.9±8.2	0	0	P>0.001		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	49.0±14.6	0	0	43.1±4.7	39.2±10.0	0	P>0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	49.0±14.6	0	0	45.8±4.5	40.5±11.3	0	P>0.05		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	49.0±14.6	0	0	44.8±8.1	38.5±10.9	0	P>0.05		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	45.8±4.5	40.5±11.3	0	44.8±8.1	38.5±10.9	0	P>0.05	P>0.05	
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	34.9±8.2	0	0	43.1±4.7	39.2±10.0	0	P<0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	45.8±4.5	40.5±11.3	0	34.9±8.2	0	0	P<0.001		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	44.8±8.1	38.5±10.9	0	43.1±4.7	39.2±10.0	0	P>0.05	P>0.05	
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	45.8±4.5	40.5±11.3	0	43.1±4.7	39.2±10.0	0	P>0.05	P>0.05	
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	44.8±8.1	38.5±10.9	0	34.9±8.2	0	0	P<0.001		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (20): Polymorphisms effect on the TG levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
TG	89.2±23.9	0	0	102±30.7	0	0	P<0.05		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	89.2±23.9	0	0	118.1±23.4	109.3±30.0	0	P<0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	89.2±23.9	0	0	131.7±34.9	125.1±41.5	0	P<0.001		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	89.2±23.9	0	0	134.4±33.2	125.2±41.7	0	P<0.001		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	131.7±34.9	125.1±41.5	0	134.4±33.2	125.2±41.7	0	P>0.05	P>0.05	
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	102±30.7	0	0	118.1±23.4	109.3±30.0	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	131.7±34.9	125.1±41.5	0	102±30.7	0	0	P<0.001		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	134.4±33.2	125.2±41.7	0	118.1±23.4	109.3±30.0	0	P>0.05	P>0.05	
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	131.7±34.9	125.1±41.5	0	118.1±23.4	109.3±30.0	0	P>0.05	P>0.05	
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	134.4±33.2	125.2±41.7	0	102±30.7	0	0	P<0.001		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (21): Polymorphisms effect on the VLDL levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
VLDL	18.2±5.4	0	0	20.7±6.1	0	0	P>0.05		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	18.2±5.4	0	0	24.5±5.7	22.2±5.9	0	P<0.05		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	18.2±5.4	0	0	26.4±7.0	25.1±8.3	0	P<0.001		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	18.2±5.4	0	0	26.9±6.7	25.2±8.3	0	P<0.001		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	26.4±7.0	25.1±8.3	0	26.9±6.7	25.2±8.3	0	P>0.05	P>0.05	
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	20.7±6.1	0	0	24.5±5.7	22.2±5.9	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	26.4±7.0	25.1±8.3	0	20.7±6.1	0	0	P<0.05		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
TT	TA	AA	TT	TA	AA	A	B	C	
26.9±6.7	25.2±8.3	0	24.5±5.7	22.2±5.9	0	P>0.05	P>0.05		
1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability			
TT	TA	AA	TT	TA	AA	A	B	C	
26.4±7.0	25.1±8.3	0	24.5±5.7	22.2±5.9	0	P>0.05	P>0.05		
2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability			
TT	TA	AA	TT	TA	AA	A	B	C	
26.9±6.7	25.2±8.3	0	20.7±6.1	0	0	P<0.001			

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (22): Polymorphisms effect on the Fe levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
Fe	81.0±19.2	0	0	122.0±29.2	0	0	P<0.001		
	Control (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	81.0±19.2	0	0	113.4±24.1	111.4±33.2	0	P<0.001		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	81.0±19.2	0	0	84.7±5.5	77.6±21.4	0	P>0.05		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	81.0±19.2	0	0	85.2±8.6	77.5±20.8	0	P>0.05		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	84.7±5.5	77.6±21.4	0	85.2±8.6	77.5±20.8	0	P>0.05	P>0.05	
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	122.0±29.2	0	0	113.4±24.1	111.4±33.2	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	84.7±5.5	77.6±21.4	0	122.0±29.2	0	0	P<0.001		
	2° PCOS(Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	85.2±8.6	77.5±20.8	0	113.4±24.1	111.4±33.2	0	P<0.001	P<0.001	
	1° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	84.7±5.5	77.6±21.4	0	113.4±24.1	111.4±33.2	0	P<0.001	P<0.001	
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	85.2±8.6	77.5±20.8	0	122.0±29.2	0	0	P<0.001		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (23): Polymorphisms effect on the Cu levels in studied groups

Groups	Control (Mean±SD)			1° UI (Mean±SD)			Probability		
Genotypes	TT	TA	AA	TT	TA	AA	A	B	C
Cu	102.0±25.1	0	0	181.5±59.0	0	0	P<0.001		
	Control (Mean±SD)			Secondary UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	102.0±25.1	0	0	188.5±43.0	160.4±47.8	0	P<0.001		
	Control (Mean±SD)			1° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	102.0±25.1	0	0	124.0±18.7	108.1±29.2	0	P<0.05		
	Control (Mean±SD)			2° PCOS (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	102.0±25.1	0	0	121.6±17.1	108.6±29.5	0	P>0.05		
	1° PCOS (Mean±SD)			2° PCO (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	124.0±18.7	108.1±29.2	0	121.6±17.1	108.6±29.5	0	P>0.05		P>0.05
	1° UI (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	181.5±59.0	0	0	188.5±43.0	160.4±47.8	0	P>0.05		
	1° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	124.0±18.7	108.1±29.2	0	181.5±59.0	0	0	P<0.001		
	2° PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	121.6±17.1	108.6±29.5	0	188.5±43.0	160.4±47.8	0	P<0.001		P<0.001
	Primary PCOS (Mean±SD)			2° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	124.0±18.7	108.1±29.2	0	188.5±43.0	160.4±47.8	0	P<0.001		P<0.001
	2° PCOS (Mean±SD)			1° UI (Mean±SD)			Probability		
	TT	TA	AA	TT	TA	AA	A	B	C
	121.6±17.1	108.6±29.5	0	181.5±59.0	0	0	P<0.001		

Probability (A): TT group 1 vs TT group 2, (B): TA group 1 vs TA group 2, (C): AA group 1 vs AA group 2.

Appendix

Appendix (24): Correlation factor of primary UI (TT) genotype between the parameters

		MDA	Cp	GSH	Cu	Fe	TG	TC	HDL	LDL	VLDL	FSH	LH	PRL	TSH	SOD
MDA	Pearson Correlation	1	.189	.241	.351	.380	.505	.569	.410	.349	.548	.572	.408	.562	.369	-.207-
	Sig.		.284	.169	.042	.027	.002	.000	.016	.043	.001	.000	.017	.001	.032	.240
Cp	Pearson Correlation	.189	1	.490	-.134-	.438	.046	.206	.222	.135	.030	.474	.350	.529	.124	.050
	Sig.	.284		.003	.450	.010	.796	.243	.208	.447	.867	.005	.042	.001	.486	.780
GSH	Pearson Correlation	.241	.490	1	-.038-	.453	.128	.471	.379	.371	.145	.309	.092	.706	-.035-	.003
	Sig.	.169	.003		.829	.007	.469	.005	.027	.031	.415	.075	.607	.000	.846	.985
Cu	Pearson Correlation	.351	-.134-	-.038-	1	.045	.222	.046	.121	-.094-	.278	.270	.260	.149	.363	.315
	Sig.	.042	.450	.829		.800	.207	.795	.495	.599	.111	.123	.137	.401	.035	.069
Fe	Pearson Correlation	.380	.438	.453	.045	1	.159	.525	.251	.474	.147	.609	.394	.558	-.075-	-.077-
	Sig.	.027	.010	.007	.800		.368	.001	.153	.005	.407	.000	.021	.001	.675	.667
TG	Pearson Correlation	.505	.046	.128	.222	.159	1	.390	.115	.150	.989	.472	.202	.133	.322	.258
	Sig.	.002	.796	.469	.207	.368		.023	.518	.397	.000	.005	.252	.455	.063	.141
TC	Pearson Correlation	.569	.206	.471	.046	.525	.390	1	.460	.911	.403	.493	.198	.507	.290	-.139-
	Sig.	.000	.243	.005	.795	.001	.023		.006	.000	.018	.003	.262	.002	.097	.434
HDL	Pearson Correlation	.410	.222	.379	.121	.251	.115	.460	1	.135	.117	.246	.174	.620	.230	-.150-
	Sig.	.016	.208	.027	.495	.153	.518	.006		.446	.511	.161	.324	.000	.192	.398
LDL	Pearson Correlation	.349	.135	.371	-.094-	.474	.150	.911	.135	1	.161	.348	.088	.301	.156	-.182-
	Sig.	.043	.447	.031	.599	.005	.397	.000	.446		.363	.044	.619	.084	.380	.303
VLDL	Pearson Correlation	.548	.030	.145	.278	.147	.989	.403	.117	.161	1	.446	.187	.155	.341	.256
	Sig.	.001	.867	.415	.111	.407	.000	.018	.511	.363		.008	.290	.381	.049	.144
FSH	Pearson Correlation	.572	.474	.309	.270	.609	.472	.493	.246	.348	.446	1	.733	.440	.369	-.102-
	Sig.	.000	.005	.075	.123	.000	.005	.003	.161	.044	.008		.000	.009	.032	.568
LH	Pearson Correlation	.408	.350	.092	.260	.394	.202	.198	.174	.088	.187	.733	1	.326	.373	-.077-

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	Sig.	.017	.042	.607	.137	.021	.252	.262	.324	.619	.290	.000		.060	.030	.666
PRL	Pearson Correlation	.562	.529	.706	.149	.558	.133	.507	.620	.301	.155	.440	.326	1	.066	-.079-
	Sig.	.001	.001	.000	.401	.001	.455	.002	.000	.084	.381	.009	.060		.710	.657
TSH	Pearson Correlation	.369	.124	-.035-	.363	-.075-	.322	.290	.230	.156	.341	.369	.373	.066	1	.036
	Sig.	.032	.486	.846	.035	.675	.063	.097	.192	.380	.049	.032	.030	.710		.840
SOD	Pearson Correlation	-.207-	.050	.003	.315	-.077-	.258	-.139-	-.150-	-.182-	.256	-.102-	-.077-	-.079-	.036	1
	Sig.	.240	.780	.985	.069	.667	.141	.434	.398	.303	.144	.568	.666	.657	.840	

Appendix

Appendix (25): Correlation factor of secondary UI (TT) genotype between the parameters

		MDA	Cp	GSH	Cu	Fe	TG	TC	HDL	LDL	VLDL	FSH	LH	PRL	TSH	SOD
MDA	Pearson Correlation	1	-.216-	.000	-.132-	-.191-	.389	-.158-	.276	-.290-	.386	-.027-	.160	-.168-	-.085-	-.220-
	Sig.		.478	.999	.667	.532	.188	.606	.361	.336	.193	.931	.602	.583	.782	.471
Cp	Pearson Correlation	-.216-	1	-.361-	-.092-	.163	.435	.209	.225	.005	.338	.016	-.378-	-.063-	-.188-	.166
	Sig.	.478		.226	.765	.594	.137	.492	.461	.986	.259	.960	.203	.837	.538	.588
GSH	Pearson Correlation	.000	-.361-	1	.070	-.382-	-.036-	-.227-	.349	-.258-	.036	-.456-	.179	.219	.221	-.608-
	Sig.	.999	.226		.819	.198	.907	.455	.242	.394	.907	.117	.558	.471	.467	.027
Cu	Pearson Correlation	-.132-	-.092-	.070	1	.257	.319	.047	-.158-	-.028-	.349	-.373-	-.121-	.010	.221	.662
	Sig.	.667	.765	.819		.397	.288	.879	.605	.927	.242	.209	.693	.975	.468	.014
Fe	Pearson Correlation	-.191-	.163	-.382-	.257	1	.187	-.203-	-.122-	-.157-	.127	.135	-.192-	.254	.144	.443
	Sig.	.532	.594	.198	.397		.541	.506	.692	.609	.680	.659	.531	.402	.640	.130
TG	Pearson Correlation	.389	.435	-.036-	.319	.187	1	-.036-	.544	-.402-	.874	-.092-	-.036-	.235	-.052-	.200
	Sig.	.188	.137	.907	.288	.541		.908	.055	.174	.000	.765	.907	.440	.866	.513
TC	Pearson Correlation	-.158-	.209	-.227-	.047	-.203-	-.036-	1	-.378-	.912	-.316-	.103	-.472-	.083	.030	.243
	Sig.	.606	.492	.455	.879	.506	.908		.203	.000	.293	.739	.104	.788	.922	.423
HDL	Pearson Correlation	.276	.225	.349	-.158-	-.122-	.544	-.378-	1	-.648-	.487	-.105-	.396	.460	-.294-	-.458-
	Sig.	.361	.461	.242	.605	.692	.055	.203		.017	.092	.733	.180	.114	.330	.115
LDL	Pearson Correlation	-.290-	.005	-.258-	-.028-	-.157-	-.402-	.912	-.648-	1	-.630-	.139	-.462-	-.080-	.112	.234
	Sig.	.336	.986	.394	.927	.609	.174	.000	.017		.021	.650	.112	.794	.715	.442
VLDL	Pearson Correlation	.386	.338	.036	.349	.127	.874	-.316-	.487	-.630-	1	-.138-	.083	.120	-.076-	.180
	Sig.	.193	.259	.907	.242	.680	.000	.293	.092	.021		.652	.789	.697	.805	.555
FSH	Pearson Correlation	-.027-	.016	-.456-	-.373-	.135	-.092-	.103	-.105-	.139	-.138-	1	-.183-	.032	-.066-	-.080-
	Sig.	.931	.960	.117	.209	.659	.765	.739	.733	.650	.652		.550	.917	.830	.795
LH	Pearson Correlation	.160	-.378-	.179	-.121-	-.192-	-.036-	-.472-	.396	-.462-	.083	-.183-	1	.352	-.670-	-.082-

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	Sig.	.602	.203	.558	.693	.531	.907	.104	.180	.112	.789	.550		.238	.012	.790
PRL	Pearson Correlation	-.168-	-.063-	.219	.010	.254	.235	.083	.460	-.080-	.120	.032	.352	1	-.335-	-.096-
	Sig.	.583	.837	.471	.975	.402	.440	.788	.114	.794	.697	.917	.238		.263	.754
TSH	Pearson Correlation	-.085-	-.188-	.221	.221	.144	-.052-	.030	-.294-	.112	-.076-	-.066-	-.670-	-.335-	1	-.066-
	Sig.	.782	.538	.467	.468	.640	.866	.922	.330	.715	.805	.830	.012	.263		.830
SOD	Pearson Correlation	-.220-	.166	-.608-	.662	.443	.200	.243	-.458-	.234	.180	-.080-	-.082-	-.096-	-.066-	1
	Sig.	.471	.588	.027	.014	.130	.513	.423	.115	.442	.555	.795	.790	.754	.830	

Appendix

Appendix (26): Correlation factor of secondary UI (TA) genotype between the parameters

		MDA	Cp	GSH	Cu	Fe	TG	TC	HDL	LDL	VLDL	FSH	LH	PRL	TSH	SOD
MDA	Pearson Correlation	1	.405	.231	.610	.663	.699	.696	.720	.348	.732	.530	.519	.691	.537	.308
	Sig.		.068	.314	.003	.001	.000	.000	.000	.123	.000	.013	.016	.001	.012	.174
Cp	Pearson Correlation	.405	1	.021	.239	.595	.456	.193	.594	-.132-	.462	.122	.431	.520	.391	.551
	Sig.	.068		.927	.297	.004	.038	.402	.004	.567	.035	.599	.051	.016	.080	.010
GSH	Pearson Correlation	.231	.021	1	.305	-.111-	.135	.464	.325	.407	.064	.170	.235	.134	.265	.127
	Sig.	.314	.927		.179	.632	.560	.034	.151	.067	.784	.462	.305	.562	.245	.583
Cu	Pearson Correlation	.610	.239	.305	1	.340	.558	.588	.302	.441	.551	.112	.318	.296	.428	.296
	Sig.	.003	.297	.179		.132	.009	.005	.183	.045	.010	.630	.160	.192	.053	.192
Fe	Pearson Correlation	.663	.595	-.111-	.340	1	.655	.461	.663	.119	.687	.332	.616	.686	.430	.450
	Sig.	.001	.004	.632	.132		.001	.036	.001	.608	.001	.141	.003	.001	.052	.041
TG	Pearson Correlation	.699	.456	.135	.558	.655	1	.432	.600	.033	.988	.518	.416	.631	.496	.602
	Sig.	.000	.038	.560	.009	.001		.050	.004	.886	.000	.016	.061	.002	.022	.004
TC	Pearson Correlation	.696	.193	.464	.588	.461	.432	1	.512	.870	.431	.449	.493	.719	.400	.389
	Sig.	.000	.402	.034	.005	.036	.050		.018	.000	.051	.041	.023	.000	.072	.081
HDL	Pearson Correlation	.720	.594	.325	.302	.663	.600	.512	1	.055	.616	.463	.433	.694	.533	.502
	Sig.	.000	.004	.151	.183	.001	.004	.018		.814	.003	.035	.050	.000	.013	.020
LDL	Pearson Correlation	.348	-.132-	.407	.441	.119	.033	.870	.055	1	.022	.205	.306	.408	.138	.119
	Sig.	.123	.567	.067	.045	.608	.886	.000	.814		.925	.373	.177	.066	.550	.609
VLDL	Pearson Correlation	.732	.462	.064	.551	.687	.988	.431	.616	.022	1	.529	.423	.653	.476	.577
	Sig.	.000	.035	.784	.010	.001	.000	.051	.003	.925		.014	.056	.001	.029	.006
FSH	Pearson Correlation	.530	.122	.170	.112	.332	.518	.449	.463	.205	.529	1	.430	.538	.314	.144
	Sig.	.013	.599	.462	.630	.141	.016	.041	.035	.373	.014		.052	.012	.166	.535
LH	Pearson Correlation	.519	.431	.235	.318	.616	.416	.493	.433	.306	.423	.430	1	.483	.218	.198

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	Sig.	.016	.051	.305	.160	.003	.061	.023	.050	.177	.056	.052		.026	.343	.389
PRL	Pearson Correlation	.691	.520	.134	.296	.686	.631	.719	.694	.408	.653	.538	.483	1	.433	.586
	Sig.	.001	.016	.562	.192	.001	.002	.000	.000	.066	.001	.012	.026		.050	.005
TSH	Pearson Correlation	.537	.391	.265	.428	.430	.496	.400	.533	.138	.476	.314	.218	.433	1	.445
	Sig.	.012	.080	.245	.053	.052	.022	.072	.013	.550	.029	.166	.343	.050		.043
SOD	Pearson Correlation	.308	.551	.127	.296	.450	.602	.389	.502	.119	.577	.144	.198	.586	.445	1
	Sig.	.174	.010	.583	.192	.041	.004	.081	.020	.609	.006	.535	.389	.005	.043	

Appendix

Appendix (27): Correlation factor of primary PCO (TT) genotype between the parameter.

		MDA	Cp	GSH	Cu	Fe	TG	TC	HDL	LDL	VLDL	FSH	LH	PRL	TSH	SOD
MDA	Pearson Correlation	1	.124	.164	.142	-.476-	.487	-.037-	.006	-.172-	.487	.028	.284	-.220-	.269	.179
	Sig. (2-tailed)		.603	.488	.550	.034	.029	.876	.981	.469	.030	.907	.225	.351	.252	.451
Cp	Pearson Correlation	.124	1	-.246-	-.252-	.057	-.272-	.411	-.530-	.543	-.271-	-.426-	-.329-	.187	-.339-	-.364-
	Sig. (2-tailed)	.603		.296	.284	.812	.247	.072	.016	.013	.247	.061	.156	.431	.144	.114
GSH	Pearson Correlation	.164	-.246-	1	.422	-.412-	.087	-.328-	.135	-.281-	.087	-.204-	.494	.159	.054	-.051-
	Sig. (2-tailed)	.488	.296		.064	.071	.715	.158	.571	.230	.716	.387	.027	.503	.821	.832
Cu	Pearson Correlation	.142	-.252-	.422	1	-.073-	.069	-.082-	.229	-.201-	.068	.108	.021	-.175-	.159	.100
	Sig. (2-tailed)	.550	.284	.064		.760	.773	.731	.331	.395	.774	.651	.929	.460	.502	.675
Fe	Pearson Correlation	-.476-	.057	-.412-	-.073-	1	-.345-	-.053-	-.315-	.087	-.344-	.162	-.374-	-.317-	.019	-.369-
	Sig. (2-tailed)	.034	.812	.071	.760		.137	.825	.176	.715	.137	.494	.104	.173	.935	.109
TG	Pearson Correlation	.487	-.272-	.087	.069	-.345-	1	-.228-	.235	-.563-	1.000	.327	.559	-.245-	-.116-	.077
	Sig. (2-tailed)	.029	.247	.715	.773	.137		.334	.319	.010	.000	.160	.010	.298	.626	.746
TC	Pearson Correlation	-.037-	.411	-.328-	-.082-	-.053-	-.228-	1	.249	.892	-.228-	-.249-	-.471-	.337	-.078-	-.347-
	Sig. (2-tailed)	.876	.072	.158	.731	.825	.334		.289	.000	.334	.289	.036	.147	.744	.134
HDL	Pearson Correlation	.006	-.530-	.135	.229	-.315-	.235	.249	1	-.058-	.234	.064	-.095-	-.025-	.083	.341
	Sig. (2-tailed)	.981	.016	.571	.331	.176	.319	.289		.810	.321	.788	.691	.917	.729	.141
LDL	Pearson Correlation	-.172-	.543	-.281-	-.201-	.087	-.563-	.892	-.058-	1	-.563-	-.341-	-.549-	.389	-.016-	-.444-
	Sig. (2-tailed)	.469	.013	.230	.395	.715	.010	.000	.810		.010	.142	.012	.090	.945	.050
VLDL	Pearson Correlation	.487	-.271-	.087	.068	-.344-	1.000	-.228-	.234	-.563-	1	.327	.559	-.245-	-.116-	.077
	Sig. (2-tailed)	.030	.247	.716	.774	.137	.000	.334	.321	.010		.160	.010	.299	.626	.748
FSH	Pearson Correlation	.028	-.426-	-.204-	.108	.162	.327	-.249-	.064	-.341-	.327	1	.022	-.686-	.167	.266
	Sig. (2-tailed)	.907	.061	.387	.651	.494	.160	.289	.788	.142	.160		.926	.001	.483	.257

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LH	Pearson Correlation	.284	-.329-	.494	.021	-.374-	.559	-.471-	-.095-	-.549-	.559	.022	1	.154	.098	.114
	Sig. (2-tailed)	.225	.156	.027	.929	.104	.010	.036	.691	.012	.010	.926		.516	.681	.632
PRL	Pearson Correlation	-.220-	.187	.159	-.175-	-.317-	-.245-	.337	-.025-	.389	-.245-	-.686-	.154	1	-.096-	-.125-
	Sig. (2-tailed)	.351	.431	.503	.460	.173	.298	.147	.917	.090	.299	.001	.516		.688	.600
TSH	Pearson Correlation	.269	-.339-	.054	.159	.019	-.116-	-.078-	.083	-.016-	-.116-	.167	.098	-.096-	1	.202
	Sig. (2-tailed)	.252	.144	.821	.502	.935	.626	.744	.729	.945	.626	.483	.681	.688		.392
SOD	Pearson Correlation	.179	-.364-	-.051-	.100	-.369-	.077	-.347-	.341	-.444-	.077	.266	.114	-.125-	.202	1
	Sig. (2-tailed)	.451	.114	.832	.675	.109	.746	.134	.141	.050	.748	.257	.632	.600	.392	

Appendix

Appendix (28): Correlation factor of primary PCO (TA) genotype between the parameters

		MDA	Cp	GSH	Cu	Fe	TG	TC	HDL	LDL	VLDL	FSH	LH	PRL	TSH	SOD
MDA	Pearson Correlation	1	.273	.155	.520	.530	.338	.513	.326	.460	.333	.369	.440	.549	.537	-.084-
	Sig. (2-tailed)		.345	.598	.057	.051	.238	.061	.256	.098	.245	.194	.116	.042	.048	.774
Cp	Pearson Correlation	.273	1	-.102-	.457	.643	-.036-	.424	.419	.628	-.037-	.512	.108	.503	.390	.323
	Sig. (2-tailed)	.345		.729	.101	.013	.903	.131	.136	.016	.901	.061	.713	.067	.167	.260
GSH	Pearson Correlation	.155	-.102-	1	.467	.462	.604	.528	.570	.309	.603	.566	.727	.518	.102	-.579-
	Sig. (2-tailed)	.598	.729		.093	.096	.022	.052	.033	.283	.022	.035	.003	.058	.728	.030
Cu	Pearson Correlation	.520	.457	.467	1	.863	.616	.873	.869	.724	.619	.716	.804	.884	.690	.070
	Sig. (2-tailed)	.057	.101	.093		.000	.019	.000	.000	.003	.018	.004	.001	.000	.006	.811
Fe	Pearson Correlation	.530	.643	.462	.863	1	.645	.859	.803	.810	.646	.858	.806	.896	.643	-.049-
	Sig. (2-tailed)	.051	.013	.096	.000		.013	.000	.001	.000	.013	.000	.001	.000	.013	.869
TG	Pearson Correlation	.338	-.036-	.604	.616	.645	1	.591	.466	.373	.999	.770	.882	.595	.485	-.158-
	Sig. (2-tailed)	.238	.903	.022	.019	.013		.026	.093	.189	.000	.001	.000	.025	.079	.589
TC	Pearson Correlation	.513	.424	.528	.873	.859	.591	1	.830	.877	.601	.645	.793	.783	.497	-.108-
	Sig. (2-tailed)	.061	.131	.052	.000	.000	.026		.000	.000	.023	.013	.001	.001	.070	.713
HDL	Pearson Correlation	.326	.419	.570	.869	.803	.466	.830	1	.664	.474	.570	.792	.904	.595	-.212-
	Sig. (2-tailed)	.256	.136	.033	.000	.001	.093	.000		.010	.087	.033	.001	.000	.025	.468
LDL	Pearson Correlation	.460	.628	.309	.724	.810	.373	.877	.664	1	.381	.625	.574	.646	.272	.115
	Sig. (2-tailed)	.098	.016	.283	.003	.000	.189	.000	.010		.179	.017	.032	.012	.348	.695
VLDL	Pearson Correlation	.333	-.037-	.603	.619	.646	.999	.601	.474	.381	1	.762	.884	.593	.480	-.150-
	Sig. (2-tailed)	.245	.901	.022	.018	.013	.000	.023	.087	.179		.002	.000	.025	.083	.608
FSH	Pearson Correlation	.369	.512	.566	.716	.858	.770	.645	.570	.625	.762	1	.760	.734	.477	-.051-
	Sig. (2-tailed)	.194	.061	.035	.004	.000	.001	.013	.033	.017	.002		.002	.003	.085	.863

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LH	Pearson Correlation	.440	.108	.727	.804	.806	.882	.793	.792	.574	.884	.760	1	.842	.577	-.316-
	Sig. (2-tailed)	.116	.713	.003	.001	.001	.000	.001	.001	.032	.000	.002		.000	.031	.270
PRL	Pearson Correlation	.549	.503	.518	.884	.896	.595	.783	.904	.646	.593	.734	.842	1	.793	-.204-
	Sig. (2-tailed)	.042	.067	.058	.000	.000	.025	.001	.000	.012	.025	.003	.000		.001	.485
TSH	Pearson Correlation	.537	.390	.102	.690	.643	.485	.497	.595	.272	.480	.477	.577	.793	1	-.046-
	Sig. (2-tailed)	.048	.167	.728	.006	.013	.079	.070	.025	.348	.083	.085	.031	.001		.876
SOD	Pearson Correlation	-.084-	.323	-.579-	.070	-.049-	-.158-	-.108-	-.212-	.115	-.150-	-.051-	-.316-	-.204-	-.046-	1
	Sig. (2-tailed)	.774	.260	.030	.811	.869	.589	.713	.468	.695	.608	.863	.270	.485	.876	

Appendix

Appendix (29): Correlation factor of secondary PCO (TT) genotype between the parameters

		MDA	Cp	GSH	Cu	Fe	TG	TC	HDL	LDL	VLDL	FSH	LH	PRL	TSH	SOD
MDA	Pearson Correlation	1	.451	-.144-	-.242-	-.071-	-.361-	-.196-	.109	-.196-	-.360-	-.198-	-.413-	-.194-	-.096-	-.094-
	Sig. (2-tailed)		.046	.545	.303	.765	.118	.408	.648	.409	.119	.402	.071	.412	.688	.692
Cp	Pearson Correlation	.451	1	-.115-	-.132-	-.044-	-.217-	.334	-.077-	.242	-.217-	.227	-.411-	-.310-	-.132-	.031
	Sig. (2-tailed)	.046		.629	.580	.853	.357	.151	.746	.304	.358	.337	.072	.183	.579	.895
GSH	Pearson Correlation	-.144-	-.115-	1	-.296-	-.240-	.176	-.247-	.135	-.089-	.176	.260	.175	.268	-.424-	.230
	Sig. (2-tailed)	.545	.629		.205	.308	.457	.295	.570	.708	.459	.269	.460	.254	.063	.329
Cu	Pearson Correlation	-.242-	-.132-	-.296-	1	.164	-.189-	.224	-.015-	.145	-.189-	-.142-	.006	.306	.643	-.154-
	Sig. (2-tailed)	.303	.580	.205		.489	.424	.342	.948	.541	.424	.552	.981	.189	.002	.517
Fe	Pearson Correlation	-.071-	-.044-	-.240-	.164	1	-.163-	.250	.103	.173	-.164-	-.076-	.138	-.190-	.356	-.002-
	Sig. (2-tailed)	.765	.853	.308	.489		.491	.289	.666	.466	.490	.750	.563	.422	.124	.993
TG	Pearson Correlation	-.361-	-.217-	.176	-.189-	-.163-	1	-.275-	-.524-	-.245-	1.000	.575	.604	.186	-.538-	.051
	Sig. (2-tailed)	.118	.357	.457	.424	.491		.241	.018	.298	.000	.008	.005	.434	.014	.831
TC	Pearson Correlation	-.196-	.334	-.247-	.224	.250	-.275-	1	.171	.817	-.274-	.063	-.460-	-.108-	.141	-.433-
	Sig. (2-tailed)	.408	.151	.295	.342	.289	.241		.470	.000	.242	.793	.041	.651	.553	.057
HDL	Pearson Correlation	.109	-.077-	.135	-.015-	.103	-.524-	.171	1	-.190-	-.524-	-.540-	-.236-	-.056-	.150	.154
	Sig. (2-tailed)	.648	.746	.570	.948	.666	.018	.470		.421	.018	.014	.316	.816	.529	.517
LDL	Pearson Correlation	-.196-	.242	-.089-	.145	.173	-.245-	.817	-.190-	1	-.245-	.227	-.440-	-.025-	.133	-.476-
	Sig. (2-tailed)	.409	.304	.708	.541	.466	.298	.000	.421		.298	.336	.052	.918	.577	.034
VLDL	Pearson Correlation	-.360-	-.217-	.176	-.189-	-.164-	1.000	-.274-	-.524-	-.245-	1	.575	.603	.186	-.539-	.050
	Sig. (2-tailed)	.119	.358	.459	.424	.490	.000	.242	.018	.298		.008	.005	.433	.014	.833
FSH	Pearson Correlation	-.198-	.227	.260	-.142-	-.076-	.575	.063	-.540-	.227	.575	1	.166	-.108-	-.328-	-.196-
	Sig. (2-tailed)	.402	.337	.269	.552	.750	.008	.793	.014	.336	.008		.483	.651	.158	.408

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LH	Pearson Correlation	-.413-	-.411-	.175	.006	.138	.604	-.460-	-.236-	-.440-	.603	.166	1	.086	-.008-	.330
	Sig. (2-tailed)	.071	.072	.460	.981	.563	.005	.041	.316	.052	.005	.483		.717	.972	.155
PRL	Pearson Correlation	-.194-	-.310-	.268	.306	-.190-	.186	-.108-	-.056-	-.025-	.186	-.108-	.086	1	-.134-	-.091-
	Sig. (2-tailed)	.412	.183	.254	.189	.422	.434	.651	.816	.918	.433	.651	.717		.572	.703
TSH	Pearson Correlation	-.096-	-.132-	-.424-	.643	.356	-.538-	.141	.150	.133	-.539-	-.328-	-.008-	-.134-	1	.132
	Sig. (2-tailed)	.688	.579	.063	.002	.124	.014	.553	.529	.577	.014	.158	.972	.572		.578
SOD	Pearson Correlation	-.094-	.031	.230	-.154-	-.002-	.051	-.433-	.154	-.476-	.050	-.196-	.330	-.091-	.132	1
	Sig. (2-tailed)	.692	.895	.329	.517	.993	.831	.057	.517	.034	.833	.408	.155	.703	.578	

Appendix

Appendix (30): Correlation factor of secondary PCO (TA) genotype between the parameters

		MDA	Cp	GSH	Cu	Fe	TG	TC	HDL	LDL	VLDL	FSH	LH	PRL	TSH	SOD
MDA	Pearson Correlation	1	.457	.605	.502	.614	.088	.668	.453	.808	.097	.535	.363	.611	.314	.193
	Sig. (2-tailed)		.101	.022	.068	.019	.766	.009	.103	.000	.742	.049	.203	.020	.275	.509
Cp	Pearson Correlation	.457	1	.195	.636	.698	.432	.582	.406	.586	.435	.622	.613	.553	.542	.171
	Sig. (2-tailed)	.101		.505	.015	.006	.123	.029	.150	.028	.120	.018	.020	.040	.045	.560
GSH	Pearson Correlation	.605	.195	1	.552	.509	.352	.596	.612	.631	.361	.441	.574	.706	.138	-.247-
	Sig. (2-tailed)	.022	.505		.041	.063	.217	.025	.020	.016	.205	.115	.032	.005	.638	.394
Cu	Pearson Correlation	.502	.636	.552	1	.830	.606	.854	.816	.790	.609	.590	.857	.847	.631	.011
	Sig. (2-tailed)	.068	.015	.041		.000	.022	.000	.000	.001	.021	.026	.000	.000	.015	.970
Fe	Pearson Correlation	.614	.698	.509	.830	1	.647	.840	.705	.869	.649	.843	.858	.870	.715	-.054-
	Sig. (2-tailed)	.019	.006	.063	.000		.012	.000	.005	.000	.012	.000	.000	.000	.004	.855
TG	Pearson Correlation	.088	.432	.352	.606	.647	1	.634	.464	.443	.999	.688	.857	.539	.477	-.173-
	Sig. (2-tailed)	.766	.123	.217	.022	.012		.015	.095	.113	.000	.006	.000	.046	.084	.554
TC	Pearson Correlation	.668	.582	.596	.854	.840	.634	1	.884	.897	.646	.583	.861	.865	.548	-.071-
	Sig. (2-tailed)	.009	.029	.025	.000	.000	.015		.000	.000	.013	.029	.000	.000	.042	.810
HDL	Pearson Correlation	.453	.406	.612	.816	.705	.464	.884	1	.763	.475	.334	.786	.884	.381	-.271-
	Sig. (2-tailed)	.103	.150	.020	.000	.005	.095	.000		.001	.086	.244	.001	.000	.180	.348
LDL	Pearson Correlation	.808	.586	.631	.790	.869	.443	.897	.763	1	.452	.626	.710	.819	.518	.084
	Sig. (2-tailed)	.000	.028	.016	.001	.000	.113	.000	.001		.105	.017	.004	.000	.058	.774
VLDL	Pearson Correlation	.097	.435	.361	.609	.649	.999	.646	.475	.452	1	.680	.860	.542	.470	-.163-
	Sig. (2-tailed)	.742	.120	.205	.021	.012	.000	.013	.086	.105		.008	.000	.045	.090	.579
FSH	Pearson Correlation	.535	.622	.441	.590	.843	.688	.583	.334	.626	.680	1	.713	.683	.618	-.102-
	Sig. (2-tailed)	.049	.018	.115	.026	.000	.006	.029	.244	.017	.008		.004	.007	.019	.729

Appendix

LH	Pearson Correlation	.363	.613	.574	.857	.858	.857	.861	.786	.710	.860	.713	1	.854	.661	-.267-
	Sig. (2-tailed)	.203	.020	.032	.000	.000	.000	.000	.001	.004	.000	.004		.000	.010	.356
PRL	Pearson Correlation	.611	.553	.706	.847	.870	.539	.865	.884	.819	.542	.683	.854	1	.549	-.343-
	Sig. (2-tailed)	.020	.040	.005	.000	.000	.046	.000	.000	.000	.045	.007	.000		.042	.231
TSH	Pearson Correlation	.314	.542	.138	.631	.715	.477	.548	.381	.518	.470	.618	.661	.549	1	-.197-
	Sig. (2-tailed)	.275	.045	.638	.015	.004	.084	.042	.180	.058	.090	.019	.010	.042		.500
SOD	Pearson Correlation	.193	.171	-.247-	.011	-.054-	-.173-	-.071-	-.271-	.084	-.163-	-.102-	-.267-	-.343-	-.197-	1
	Sig. (2-tailed)	.509	.560	.394	.970	.855	.554	.810	.348	.774	.579	.729	.356	.231	.500	

الخلاصة

صممت هذه الدراسة لتشخيص الدور الممرض هرمونات الخصوبة , للإجهاد التأكسدي, مضادات الاكسدة, مستويات الدهون, بعض العناصر النزرة , وتعدد اشكال جين الكلوتاثايون روداكتيز. لقد تضمنت الدراسة ثمانية وستون امرأة تعاني من العقم غير المفسر (UI) وكذلك ثمانية وستون امرأة تعاني من متلازمة تكيس المبايض (PCOS) تتراوح أعمارهن بين (35-20) سنة, تم اخذ عينات التي تضمنتها الدراسة من وحدة العقم في مستشفى الحسين التعليمي في محافظة ذي قار . كما تم تقسيم نساء العقم الغير مفسر الى مجموعتين هما مجموعة نساء العقم الغير مفسر الاولي والثانوي ($1^{\circ}UI$, $2^{\circ}UI$) كما تم تقسم نساء عقم تكيس المبايض الى مجموعتين هما عقم تكيس المبايض الاولي والثانوي ($1^{\circ}PCO$, $2^{\circ}PCO$). كذلك تضمنت الدراسة 34 امرأة خصبة صحيحة بنفس عمر النساء العقيمات كمجموعة سيطرة.

أظهرت النتائج وجود زيادة معنوية في هرمون الحليب (PRL) , هرمون تحفيز الغدة الدرقية (TSH) وهرمون التبيوض (LH) في مصل مجموعتي النساء ذوات عقم (PCOS) مقارنة مع النساء ذات العقم غير المفسر (UI)، و زيادة معنوية في مستويات مالون داي الديهايد (MDA) في جميع مجاميع العقم مقارنة بمجموعة السيطرة . في حين انخفضت مستويات المصل لمضادات الأكسدة الكلوتاثايون (GSH)، سوبراوكسايد دسميوتيز (SOD) وسيرولوبلازمين (Cp) بشكل ملحوظ في جميع مجاميع العقم بالمقارنة مع مجموعة السيطرة .كما أظهرت النتائج وجود زيادة معنوية (TG, VLDL, LDL, TC) وانخفاض في مستويات (HDL) في مصل جميع مجاميع العقم مقارنة بمجموعة السيطرة. كذلك هناك زيادة كبيرة في (TG, LDL, TC) وانخفاض في (HDL) في مصل مجموعتي نساء عقم ($1^{\circ}PCO$), ($2^{\circ}PCO$) بالمقارنة مع مجموعتي ($1^{\circ}UI$) و($2^{\circ}UI$). كما لوحظ ان مستويات الحديد والنحاس تزيد بشكل ملحوظ في مصل مجموعتي العقم الغير مفسر بالمقارنة مع مجاميع العقم PCOS ومجموعة السيطرة . بينما لم تكن هناك فروق معنوية في مستويات الحديد بين مجموعتي عقم PCOS و مجموعة السيطرة .كما ازدادت مستويات النحاس بشكل ملحوظ في مصل مجموعتي عقم PCOS مقارنة مع مجموعة السيطرة .

النتائج التي تم الحصول عليها من تفاعل البوليميراز المتسلسل (PCR) للكشف عن الجين (GSR) في عينات من الحمض النووي المستخلصة من النساء العقيمات المصنفة على أنها ($1^{\circ}UI$, $2^{\circ}UI$, $1^{\circ}PCO$, $2^{\circ}PCO$). أعطى التضخيم لجين (GSR) نتائج إيجابية لجميع

العينات ، كما كشف التضخيم المنتج تعدد الأشكال لاثنتين من الوزن الجزيئي 250 bp . وقد تم تحليل نتائج التسلسل من خلال معلومات المركز العالمي للتكنولوجيا الحيوية (NCBI) على شبكة الإنترنت في ([http:// www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) و Bio Edit program لكشف عن تعدد الأشكال في جين (GSR) لمجموعة 2°UI فقد وجدت 8 طفرات من بينهم 5 طفرات غرس (عرس القاعدة النيوكليوتيدية الثايمين T) أظهرت درجة التوافق 99% والمسجل (217) بايت و المتوقع 53-6e , كما أظهرت 3 طفرات تحول (يشير إلى تعويض الثايمين بدل الادنين) (A ↔ T) أظهرت درجة التوافق 98% والمسجل (219) بايت و المتوقع 53-2e اما المجاميع (1°PCO و 2°PCO) ، وجدت فيها الطفرات بين 9 insertion (يشير إلى غرس القاعدة النيوكليوتيدية الثايمين T) أظهرت التطابق 99% والمسجل (228) بايت (123) و المتوقع 56-3e و 4 طفرات transversion يشير إلى تعويض الادنين بدل الثايمين (A ↔ T) أظهرت درجة التوافق 98% (219) بايت و المسجل (118) و المتوقع 53-2e في العقم المفسر الاول (1°PCO) والعقم المفسر الثانوي (2°PCO) . كما بينت النتائج وجود تأثير لتعدد الأشكال وتوزيع النمط الوراثي على مستوى مؤشر الاكسدة في جميع مجاميع العقم المدروسة (1°UI 2°UI, 1°PCO, 2°PCO) كما كان هناك تأثير لتعدد الأشكال (GSR) والتنميط الجيني له على العلامات الكيميائية الحيوية المدروسة (LH, TSH, PRL , Fe, Cu, TC, TG, VLD, LDL, Cp, SOD, GSH) وخلصت هذه الدراسة إلى وجود اضطراب في التوازن بين الاكسدة و مضادات الأكسدة في مصل النساء المصابات بالعقم المفسر ونساء عقم PCOS كما تقترح الدراسة ان هناك علاقة بين مستويات الحديد والنحاس المرتفعة والعقم غير المفسر. كذلك بين الكشف عن تعدد الأشكال في جين (GSR) وجود طفرات في تردد الاليل (TA) في جين (GSR) يشترك مع زيادة خطر عقم الغير المفسر الثانوي و تكيس المبايض الاول والثانوي كذلك هناك تأثير لتردد الاليل و تعدد اشكال التنميط الجيني لجين (GSR) على العلامات البيوكيميائية.

October, 2017 A.D.

Muharram, 1439

A.H.



وزارة التعليم العالي والبحث العلم

جامعة ذي قار

كلية العلوم

دراسة سريرية جينية لحالة أكسدة - مضادات الأكسدة لدى النساء المصابات بالعم غير المفسر و النساء المصابات بعم تكيس المبيض

الاطروحة

مقدمة إلى مجلس كلية العلوم / جامعة ذي قار كجزء من متطلبات نيل درجة
الدكتوراه فلسفة في الكيمياء الحياتية السريرية

من قبل

خناء عودة حسين الجبوري

بكالوريوس الجامعة المستنصرية (2003)

ماجستير جامعة ذي قار (2013)

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تشرين الثاني 2017 م

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