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Ministry of Higher Education
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Gene Expression of Superoxide Dismutase and Determination of Other Antioxidants associated with Polycystic Ovarian Syndrome

A thesis
Submitted to the Council of the College of Science at University of Babylon in Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy of Science in Biology
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الخلاصة

متلازمة المبيض المتعدد الكيسات (PCOS) هي واحدة من اضطرابات الغدد الصماء الشائعة التي تصيب العديد من النساء في سن الإنجاب، تبدأ هذه المتلازمة من وقت حدوث الحيض حتى سن اليأس وعادة ما تسبب العقم في جميع أنحاء العالم. كشفت العديد من الدراسات أن علامات الجهد التأكسدي تزداد بشكل كبير في المرضى الذين يعانون من متلازمة تكيس المبايض مقارنة بالعوامل الطبيعية وتعتبر بمثابة حافز محتمل للتسبب في الإصابة بمتلازمة تكيس المبايض .

هدفت الدراسة الحالية إلى تقييم نشاط إنزيمات SOD في عينات الدم (البلازما وكريات الدم الحمراء) وارتباطها بالتعبير الجيني SOD1 و SOD2في المرضى المصابين بمتلازمة تكيس المبايض. شملت الدراسة الحالية 75 أنثى تم تأكيد إصابتها بمتلازمة تكيس المبايض. تم جمع العينات من عيادة أمراض النساء الخاصة من نوفمبر 2019 حتى أكتوبر 2021 واستند التشخيص السريري لمتلازمة المبيض المتعدد الكيسات إلى معايير روتردام 2003. تتألف المجموعة الضابطة من 75 امرأة تتمتع بصحة جيدة مع دورة شهرية منتظمة، ولا توجد علامة على فرط الأندروجين، ولديها مستويات هرمونية طبيعية. تراوحت الأعمار في كلا المجموعتين بين (20 - 45) سنة. تم ملء استمارة الاستبيان لكل مريض وضابطة بما في ذلك التاريخ الاجتماعي والديموغرافي والصحي بالإضافة إلى المعايير السريرية مثل مؤشر كتلة الجسم والمستوى الهرموني للهرمونات (LH) وهرمونات تحفيز الجريب (FSH) .تم استبعاد فرط بروﻻكتين الدم ، وأمراض الغدة الدرقية ، وتضخم الغدة الكظرية الخلقي غير التقليدي ، والنساء اللواتي تناولن موانع الحمل أو التنشيط ، والنساء المدخنات أو المصابات بارتفاع ضغط الدم ، ومرض السكري .

تتضمن الدراسة الحالية جزئيين: دراسة الكيمياء الحيوية والجزيئية. اشتملت الدراسة البيوكيميائية على قياس أنشطة إنزيم Cu /znSOD و MnSOD و Catalase ، وحالة مضادات الأكسدة الإجمالية (TAC) وحالة المؤكسدات الكلية (TOS) في البلازما وكريات الدم الحمراء لكل عينة من مرضى متلازمة تكيس المبايض والضوابط. تم فحص تأثير عقار الميتفورمين على هذه المعايير في جميع العينات. كما تم تحديد مستويات هرمون LH و FSH في عينات المصل .بينما تضمن الجزء الثاني الدراسة الجزيئية التي تناولت القياس الكمي للتعبير الجيني لـ SOD1 و SOD2باستخدام تقنية النسخ العكسي الكمي لتفاعل البوليميراز المتسلسل (RT-qPCR).تم قياس التعبير في هذه الجينات وتحليلها باستخدام إنزيماتها النشطة لتحديد ارتباطها بالمرض .أظهرت نتائج هذه الدراسة وجود فروق ذات دلالة إحصائية بين مجموعات PCOS والضابطة، وازدادت الفعالية الأنزيمية لل Cu\zn SOD و MnSOD بشكل كبير في البلازما وكريات الدم الحمراء

للمرضى مقارنة بالضوابط ($P \leq 0.01$) ، بينما انخفض نشاط إنزيم الكاتلايز في البلازما وكريات الدم الحمراء ($P \leq 0.05$) .زاد مستوى TOS بشكل معنوي في جزء البلازما ($P \leq 0.01$) ، بينما انخفض TAC في البلازما وكريات الدم الحمراء لنساء متلازمة تكيس المبايض عند ($P \leq 0.01$)

أظهرت المجموعة المعالجة بعقار الميتفورمين انخفاضًا في نشاط إنزيم Cu/znSOD ونشاط إنزيم الكاتلايز ومستوى TOS ، بينما زاد نشاط MnSOD و TAC. أظهر الارتباط بين متغيرات مضادات الأكسدة في مرضى متلازمة تكيس المبايض ارتباطًا إيجابيًا معنويًا بين الكاتلايز و Cu/znSOD($r = 0.219$,$P < 0.05$)، وارتباط معنوي سالب بين(Cu / znSOD (TOS, و(TAO , MnSOD)و .(Cu,Vit E).ازداد النحاس بشكل كبير في النساء المتلازمة بينما انخفض حمض الأسكوربيك وألفا توكوفيرول .يُظهر الفحص الهرموني ارتفاع هرمون LH مع انخفاض هرمون FSH في مرضى متلازمة تكيس المبايض، في حين أن SOD1 و SOD2 لهما علاقة إيجابية مع هرمون LH ، و الكاتلايز له علاقة سلبية

.كشفت النتائج عن وجود علاقة معنوية بين مرض متلازمة تكيس المبايض وارتفاع نشاط الإنزيم ومستويات التعبير عن SOD1 و .SOD2وكان العلاج باستخدام عقار الميتفورمين مرتبطًا بشكل كبير بمستوى أعلى من النشاط والتعبير فيSOD2 ، مع خفض تعبير SOD1 أيضًا ، تم العثور على ارتباط إيجابي بين جين LH وSOD1 ، SOD2 ، في حين تم إظهار ارتباط سلبي بين SOD1، جينSOD2 ، وهرمون .FSH وفقًا لما سبق ذكره، يمكن اعتبار معلمات / Cu znSODو MSOD و Catalase و TAC و TOS في عينات البلازما مؤشرات حيوية واعدة لمتلازمة المبيض المتعدد الكيسات. علاوة على ذلك، يمكن اعتباره علامة بيولوجية لتقييم تطور اضطراب التمثيل الغذائي الذي قد يتسبب في منع الإباضة مما قد يؤدي إلى العقم

Summary

Polycystic ovary syndrome (PCOS) is one of the common endocrine disorders affecting many women at reproductive age that start from menarche until menopause and commonly causes infertility around the world. Many investigations have revealed that oxidative circulating markers are significantly increased in patients with PCOS compared with normal and are considered as a potential inducement of PCOS pathogenesis.

The present study aimed to evaluate the activity of Superoxide dismutase enzymes (SOD enzyme) in the blood samples (plasma and erythrocytes) and their association with *SOD1* and *SOD2* gene expression in patients diagnosed with PCOS. The current study included 75 females clinically confirmed with PCOS. Samples were collected from a private gynecologist clinic from November 2019 until October 2021 and the clinical diagnosis of polycystic ovary syndrome was based on the Rotterdam 2003 criteria. The control group comprises 75 healthy women with a regular menstrual cycle, no sign of hyperandrogenism, and have normal hormonal levels. The age in both groups was ranged between (20 to 45) years. The questionnaire form has been filled for each patient and control including socio-demographic and health history as well as clinical criteria such as body mas index and hormonal level of luteinizing (LH), and follicle-stimulating (FSH) hormones. Hyperprolactinemia, Thyroid disease, non-classical congenital adrenal hyperplasia, women taken contraceptives or activation, smoked women or have hypertension , and diabetes all were excluded.

The present study was carried out in two parts: Biochemical and molecular study. The biochemical study was involved the measurement of Cu/znSOD, MnSOD, and Catalase enzyme activities, Total antioxidants status (TAC) and Total oxidants status (TOS) in the plasma and erythrocyte

for each sample from PCOS patients and controls. The effect of metformin drug on these parameters has been investigated in all samples. LH and FSH hormone levels in the serum samples were also determined.

The second part was included the molecular study that dealing with quantification of *SOD1* and *SOD2* gene expression by using quantitative reverse transcription polymerase chain reaction (RT-qPCR) technique. The expression of these genes was measured and analyses with their enzymes activities to define their association with the disease.

The results of this study showed highly significant differences between PCOS and control groups, Cu/znSOD and MnSOD were significantly increased in the plasma and erythrocyte of patients than in controls ($P \leq 0.01$), while catalase enzyme activity was decreased in the plasma and erythrocyte ($P \leq 0.05$).TOS level was significantly increased in the plasma fraction ($P \leq 0.01$), whereas TAC was reduced in the plasma and erythrocyte of PCOS women at ($P \leq 0.01$).

The metformin-treated group showed a decrease in Cu/znSOD , catalase enzyme activity and TOS level .While MnSOD activity and TAC were increased .The Correlations between antioxidant parameters in PCOS patients showed a significant positive correlation between catalase and Cu/znSOD ($r=0.219$, $P<0.05$), and negative significant correlation between (Cu/znSOD,TOS),(MnSOD ,TAO) and (Cu ,Vit E) . Copper was significantly increased in PCOS women while ascorbic acide and alpha-tocopherol were decreased.

Hormonal assay show elevated LH hormone with reduced FSH hormone in PCOS patients, whereas SOD1and SOD2 have a positive correlation with LH hormone ,while catalase have a negative correlation .on the other hand, FSH hormone have a negative correlation with SOD2 and catalase enzyme activity and positive relationship with SOD1.

The results revealed a significant association between PCOS disease

and higher enzyme activity and expression levels for *SOD1* and *SOD2* . Treatment with Metformin drug was significantly related to a higher level of activity and expression of *SOD2*, while lowering the expression of *SOD1*.Also, There was a positive association was found between LH and *SOD1*, *SOD2* gene, whereas a negative correlation was shown between *SOD1*, *SOD2* gene, and FSH hormone.

According to the previously mentioned, Cu/znSOD, MSOD, Catalase, TAC, and TOS parameters in the plasma samples can be considered promising biomarkers for the polycystic ovarian syndrome. Furthermore, it may consider as a biomarker to evaluate the progression of metabolic disturbance that may cause t h e prevention of ovulation which may lead to the infertility

Appendix

Questionnaire for female patients

File number.....
Name ……. Age .....
Height …….cm, weight…. ..... kg
City origin …….
Married? ……
Number of children ……..
History of infertility …….
Have you history of abortion?
Have you been diagnosed with other disease(diabetes or Hypertension)?
Have you taken any drug?
Have you take metformin?
Are the family member have infertility problem?
Menstrual cycle? Regular …… Or irregular ……
PCOS diagnosed by:

1. Ultra sound
2. FSH……, LH……
3. TSH ……
4. Other symptoms ?Hirsutism ?Acne ?Obisity ?

Husband' health ?
Education level?
Feeding habit?
Stress?

Appendix (1): Questionnaire for female patients.

Appendix (2) : Correlation between antioxidant parameters in control group.

Parameter	Cu\zn SOD	Mn SOD	Catalase	TOS	TAC	Vit E	Vit C	Cu
Cu\zn SOD		0.165	0.036	0.205	0.041	0.081	0.028	-0.096
MnSOD	0.165		-0.019	0.161	-0.115	0.063	-0.041	0.085
Catalase	0.036	-0.019		-0.112	0.365	0.102	-0.073	-0.100
TOS	0.205	0.161	-0.112		0.104	-0.06	-0.002	-0.012
TAC	0.041	-0.115	0.365	0.104		-0.10	-0.092	-0.137
Vit E	0.081	0.063	0.102	-0.066	-0.106		0.344	0.078
Vit C	0.028	-0.041	-0.073	-0.002	-0.092	0.344		-0.019
Cu	-0.096	0.085	-0.100	-0.012	-0.137	0.078	-0.019	

Values are presented as Pearson correlation. The values represented with * and ** represent 2-tailed correlation significant at 0.05 and 0.01 levels, respectivel

Conclusions

1. Women with Polycystic ovarian syndrome exhibited increased Cu/znSOD, MnSOD enzyme activity with decreased catalase activity compared to controls. Suggesting that the byproduct of oxidative damage is expected to be raised in women with PCOS
2. The enzyme activity was more obvious in plasma fraction than in erythrocyte; thus, it can be considered as a biomarker for a polycystic ovarian syndrome
3. An increase in TOS level (in the plasma and erythrocyte samples) was found in women with PCOS, suggested that women with PCOS are under oxidative stress and supports the concept that oxidative stress is involved in PCOS pathophysiology.
4. A significant increase was found in the TAC level in the plasma and erythrocyte samples of PCOS patients than in normal women, in parallel with increased oxidative stress.
5. Increased zinc level in the polycystic ovary syndrome group with decreased ascorbic acid and tocopherol may be associated with oxidative stress within the body that puts the antioxidant defense system in a hyperactive state to compensate for this stress.
6. Treatment with Metformin drug led to reduc the oxidant status and total antioxidant capacity as this drug is responsible for reducing circulating insulin and increasing the glucose uptake by peripheral tissues.
7. SOD1 and catalase enzyme activity were significantly reduced in erythrocytes and plasma fractions due to reduced oxidative stress, while SOD2 showed a distinct pattern with enhanced enzyme activity in plasma and erythrocyte samples after metformin administration.

8. A significant association between PCOS and higher expression levels in *SOD1* and *SOD2* gene with increased activity was reprted. Treatment with Metformin drug was significantly related to a higher level of activity and expression of SOD2 , while lowering the expression of SOD1. These findings can open a new viewpoint in understanding the pathogenesis of PCOS, suggesting that OS might be involved in developing this syndrome.
9. There was a relationship between LH and FSH levels and antioxidant enzyme status; A positive association was found between LH and *SOD1*, *SOD2* gene, whereas a negative correlation was shown between *SOD1, SOD2* gene, and FSH hormone. These results provide supportive evidence that LH and FSH hormones in PCOS may contribute to enhanced antioxidant enzymes and oxidative stress.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders of women at reproductive age and the major cause of anovulatory infertility (Morin-Papunen *et al*., 2012). Most characteristics include hyperandrogenism, oligomenorrhea, and polycystic ovarian morphology (PCO). Hirsutism, acne, insulin resistance, obesity, impaired glucose tolerance, type II diabetes, and high blood luteinizing hormone (LH) levels are also symptoms of this illness. Furthermore, women with PCOS are at a higher risk of cardiovascular events. (criteria & syndrome, 2004)

PCOS is commonly related to Insulin resistance (IR), a physiological condition in which cells fail to respond to insulin's effects, resulting in dysfunctions of glucose transport and utilization. IR considers as a biomarker of women with PCOS and is usually related to oxidative stress (OS) (Savic-Radojevic e*t al.*, 2015). Hyperglycemia and higher levels of free fatty acid lead to increase reactive oxygen species (ROS) production. in which excess glucose and free fatty acid oxidation in the cell cause accumulation of reducing metabolites like pyruvic acid and acetyl co-enzyme A in the mitochondria and enhancing the activity of the electron transport chain, resulting in increased ROS generation (Zuo *et al*., 2016).

Antioxidants are a group of molecules that can reduce the destructive effects of free radicals found in all organisms. They are two types: either enzymatic antioxidants like superoxide dismutase, catalase, glutathione peroxidase (GPx), and glutathione reductase (GR) or non-enzymatic antioxidants such as glutathione (GSH), α-tocopherol (vitamin E), ascorbate (vitamin C), and β-carotene (Fang *et al.*, 2002).

These antioxidants have been reported to have an important role in the female reproductive system and in the pathogenesis of female infertility (Agarwal *et al.,* 2005b).

The most important antioxidant enzyme is the superoxide dismutase (SOD) which is involved in the dismutation reaction of harmful ROS, $O2^{\bullet-}$. The products of this reaction include molecular oxygen (O2) and hydrogen peroxide (H_2O_2) that can catalyze by either GPX or catalase into H_2O (McCord *et al.*, 1971). Three different types of SOD have been recognized in organisms. Cu/Zn SOD (SOD1) is located in the cytoplasm and accounts for approximately 50–80% of the total SOD activity, MnSOD (SOD2) it is the only antioxidant enzyme that presents in mitochondria (a major site for the production of ROS during normal cellular metabolism), and EC SOD (SOD3) a glycoprotein located in the extracellular space (Weisiger & Fridovich, 1973).

ROS and antioxidants play a physiological role in folliculogenesis, oocyte maturation, luteal regression, and fertilization in the female reproductive system (Agarwal et al., 2005a). However, oxidative stress has been linked to reduced oocyte fertilization (Jančar *et al.,* 2007). ROS imbalance can be caused by environmental or genetic changes in antioxidant enzyme activity (Beyer *et al.,* 1991). Previous research has shown that SOD enzyme activity is related to oocyte quality and may impact the outcome of assisted reproductive procedures (Borowiecka *et al.,* 2012). A genetic investigation conducted by Ruiz-Sanz recently supported this hypothesis (Ruiz-Sanz et al., 2011)

SOD1 and *SOD2* genes are located on chromosomes 21q22.11 and 6q25.3 and comprise five exons, respectively. Both are widely expressed in all human tissues (Miao & Clair, 2009). Several investigations have revealed that ROS level is significantly increased in patients with PCOS compared

to healthy women (Agarwal *et al*., 2005a; Jančar *et al*., 2007; Murri *et al*., 2013). Though ROS is considered as a potential inducer of PCOS pathogenesis (Murri *et al.,* 2013), it is unknown whether antioxidant defense mechanism-related genetic variants are associated with the risk of PCOS.

The regulation of SOD genes is critical in balancing the levels of ROS. The control of SODs at both the expression and activity levels contributes in the up and down regulation of ROS level (Wang *et al.*, 2018). SODs provide significant roles in addition to the fundamental role of SOD2 in aerobic survival (Kurutas, 2015). Studying how the expression of SOD genes effect on this physiological status might aid in understanding human disorders and developing therapeutic therapies

Aim of the study

The aim of this study was to evaluate the activity of SOD enzymes in the blood samples and their association with *SOD1* and *SOD2* genes expression in patients diagnosed with PCOS; the aim of this study was carried out by the following objectives:

1. Determine the anthropometric and pathological characteristics of all patients
2. Measure Cu/zn SOD, MnSOD, and catalase enzyme activity in the plasma and erythrocyte of PCOS and controls groups.
3. Evaluate TAC and TOS in the plasma and erythrocyte of PCOS and controls groups.
4. Determine the gene expression of *SOD1 and SOD2* genes using the Real-time quantitative PCR technique (RT-qPCR).
5. The study will also investigate any possible relationship of these parameters with clinical and hormonal factors (LH and FSH hormones).

6. Compare the effects of metformin, an alternative medication to PCOS patients, in enzyme activity and its expression along with determine the benefits of this medication in improving the oxidative status.
7. Determine copper, ascorbic acid and alpha tocopherol level in patients and control.

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2. Literature Review

2.1. Ovarian function

The ovary is the essential organ of the female reproductive system. It has two main functions: the production of gametes or oocytes and the secretion of signalling and regulatory substances, including those that influence other parts of the reproductive system in controlling female maturation, gamete production, regularity of cycle and behaviour, and those, which support embryo development, gestation, and lactation. The ovary has two structural regions: the outer cortex contains follicles at various stages of development together with structures derived from follicles, while the inner medullar consists mainly of stromal tissues and vascular elements (Figure 2.1)(Sirotkin & Luck, 2012)

Several factors can affect ovarian morphology and physiology, like polycystic ovary syndrome, which is further exacerbated by obesity, in which Ovarian volume and follicle number increase in women with PCOS(Alsamarai *et al*., 2009). Furthermore, the incidence of uterine myomas (fibroids) and endometriosis (a progressive pelvic inflammatory disorder) is the most common gynaecological diseases affecting women's quality of life and ovaries function (Uimari *et al*., 2021). In addition, several genetic factors may limit the “ovaryan reserve” an expression of the total number of oocytes within the ovary; in which many genes are involved in ovary development and function and many more are vital participants in the implicated pathways(Pelosi *et al*., 2015). Moreover, environmental factors may also influence reproduction and ovary development. There are suggestions that dioxin exposure is linked to endometriosis, phthalate exposure may affect ovarian reserve, and bisphenol A can interfere with oocyte progress and maturation(Hart, 2016).

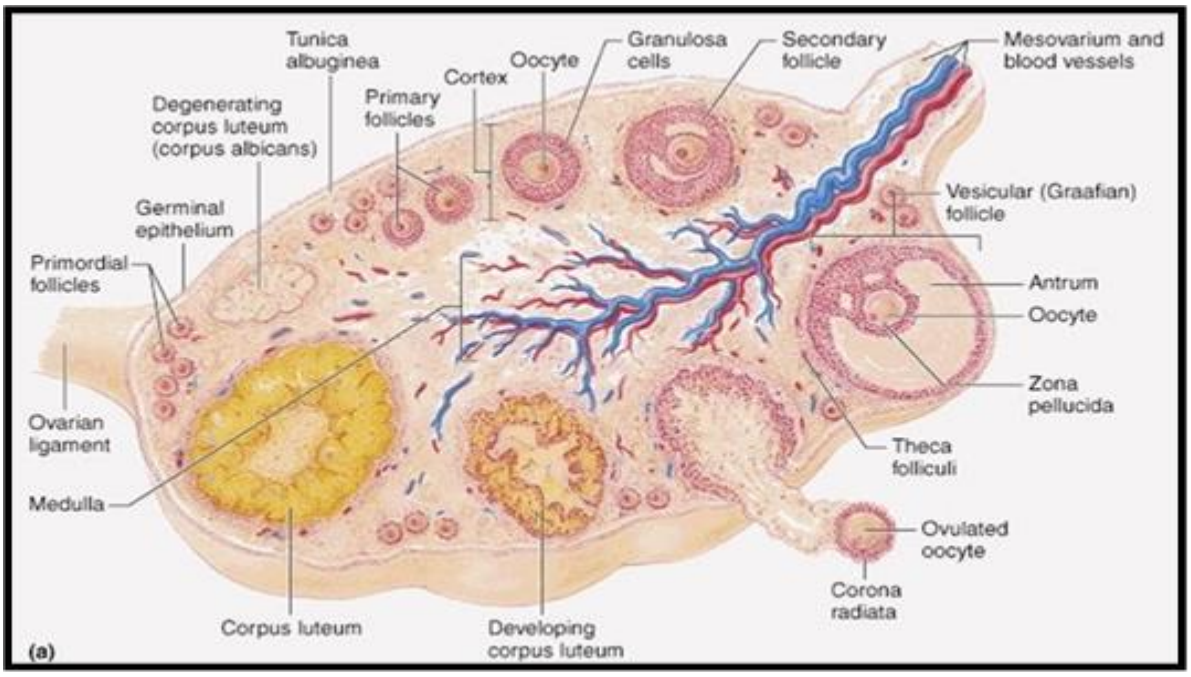


Figure (2.1): The anatomy of the ovary (L Mescher, 2016)

2.2. Polycystic ovarian syndrome (PCOS)

2.2.1. Criteria for diagnosis of PCOS

Polycystic ovarian syndrome is one of the most common endocrine disorders of women of reproductive age and the major cause of ovulatory infertility (Joham *et al*., 2015). It was defined as the change of ovarian morphology by Chereau in 1844 (Chéreau, 1844). The European Society established the diagnostic criteria for Human Reproduction and Embryology (ESHRE) and the Americans Society of Reproductive Medicine (ASRM) in 2003 based on extensive studies during the last decades, which is then so-called Rotterdam Consensus Criteria(Fauser *et al*., 2012). It was first described by Stein and Leventhal as a syndrome of oligomenorrhea and polycystic ovaries, which was variably attended by hirsutism, acne, and obesity (Stein, 1935). Later, biochemical and radiographic imaging was assumed to assist in diagnosis (Bachanek *et al*., 2015).

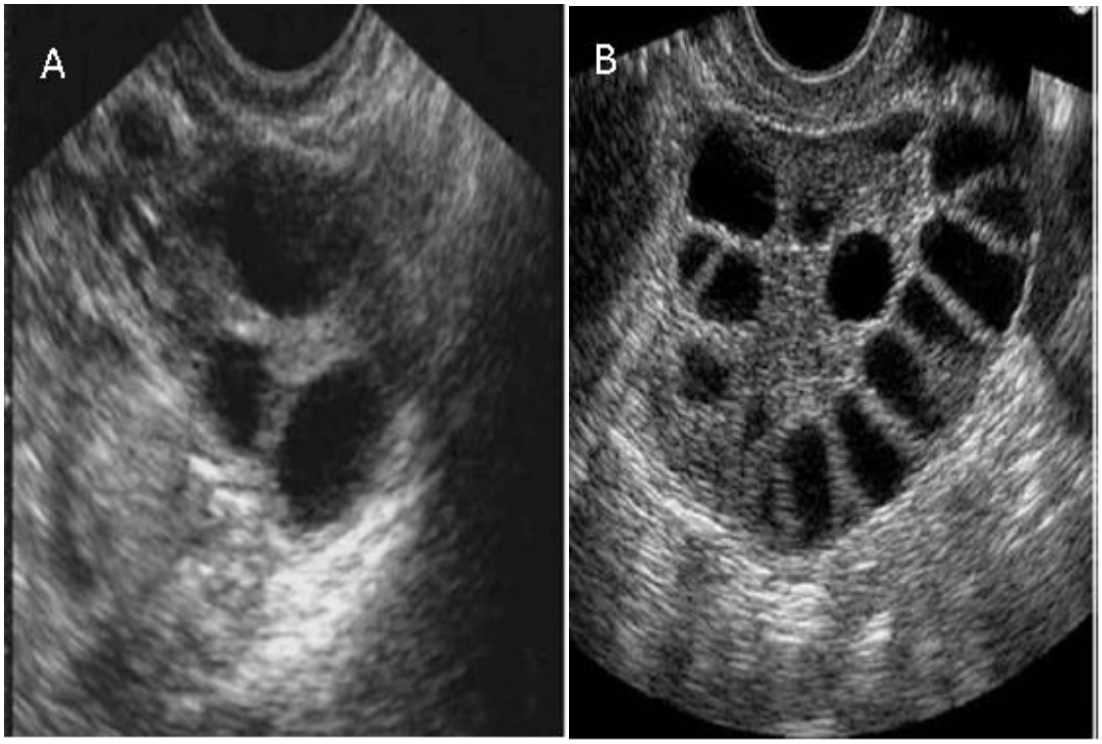
In early 1970, the scientific community studied increased serum levels of LH elevated LH/FSH ratio, and changed function in the hypothalamic–pituitary–ovarian axis that can be associated with PCOS pathology (Rebar *et al*., 1976) Moreover, The ultra-sonographic finding of polycystic ovaries was first used in 1981 as one diagnostic criterion of PCOS in the visualizing and diagnosis of polycystic ovaries (Swanson *et al*., 1981).

Then in 2003, the Rotterdam Criteria used ultrasound as a new criterion to be added to the two previous criteria. The Europeans Society of Human Reproductions and Embryology/American Society for Reproductive Medicine Rotterdam consensus developed the diagnosis of PCOS, requiring two of three features: anovulation or oligo ovulation, clinical and/or biochemical hyper androgens and polycystic ovarian morphology seen on ultrasound. Finally, In 2006, the androgen Excess and PCOS Society (AE-PCOS) underlined PCOS to be regarded as a condition of androgen excess and defines the syndrome as hyperandrogenism together with oligo/anovulation and polycystic ovaries, which is the third criterion (Azziz *et al.,* 2006).

Exclusion of other androgen excess conditions should be excluded such as non-classical congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumors, hyperprolactinemia, thyroid diseases, drug-induced androgen and other causes of oligomenorrhea or anovulation (Spritzer, 2014). Also, the National Institutes of Health Criteria (NIH) have been proposed some criteria for the diagnosis of PCOS in 1990 that include the only presence of clinical and biochemical hyperandrogenism and oligomenorrhea anovulation (Zawadeski & Dunaif, 1992)**.**

The syndrome acquired its name due to multiple ovarian cysts diagnosed using ultrasound by the presence of eight or more follicular cysts, usually with an average size of 2-9 mm in diameter arranged around a dense stroma within the PCOS ovary. The combination of multiple follicles and an

increased amount of stroma contribute to the overall increase in the ovarian size (ovarian volume >10cm^3), which is 1.5 to 3 times larger than normal (Balen, 2004) (Figure 2.2)



Normal *ova*ry **Polycystic ovary**

Figure (2.2): Comparison of normal and polycystic ovary (Hiremath & Tegnoor, 2013).

2.2.2. Clinical features of PCOS

Polycystic ovary syndrome (PCOS) is an endocrine disorder that has multiple potential etiologies and variable clinical symptoms in women of reproductive age (Ercan *et al.,* 2013).clinical and Biochemical hyperandrogenism of ovarian origin and to a lesser extent adrenal is evident for about 60–80% of PCOS patients, resulting in one of the main features of this syndrome (Franks, 2006). Ovarian hyperandrogenism is due to a defect in the intrinsic steroid synthesis in ovarian thecal cells which results in high levels of LH and low levels of FSH which consider the most stable and obvious diagnostic feature of PCOS that is evaluated clinically by

hirsutism (Hirsutism , excessive growth of terminal hair on the face and body of a female in a typical male pattern distribution ,acne and alopecia(Diamanti-Kandarakis *et al.*, 1999; Somani *et al.*, 2008). A recent report by the Androgen Excess PCOS Society analyzed 18 studies from 1983 to 2007, including 6281 women with PCOS, and found that 74.7% of women have hirsutism(Azziz *et al.*, 2009). Hirsutism in these women is generally more severe in the context of obesity, particularly the abdominal phenotype (Carmina, 2006; Gambineri et al., 2002; Wild, 2004).

Menstrual abnormality is the most important feature of the polycystic ovarian syndrome, which includes oligomenorrhea (infrequent menses with less than nine menstrual periods per year), amenorrhea (absence of menstruation for > 6 months without being pregnant), and irregular bleeding (loss of the cyclic menstrual pattern) (Hoeger *et al*., 2014).

PCOS associated with an increased risk of metabolic disorders that include insulin resistance(IR) and hyperinsulinism, diabetes mellitus, cardiovascular disease ,dyslipidemia, and endometrial carcinoma(Azziz et al., 2004). Another common feature of PCOS is obesity, Approximately 50% of PCOS women are overweight or obese, which plays a pathogenetic role in the development of the syndrome in susceptible individuals, obese PCOS women have elevated hyperandrogenism and related clinical features like hirsutism, anovulation and menstrual abnormalities than normal-weight PCOS women. This picture tends to be more distinct in obese PCOS women with the abdominal phenotype (Gambineri *et al.*, 2002).Along with impaired metabolic and reproductive features, Stress and depression are considered high-risk factors. This high level of stress and anxiety in patients with PCOS may be due to various reasons such as obesity, hirsutism, alopecia, and infertility (Sadeeqa *et al.*, 2018)

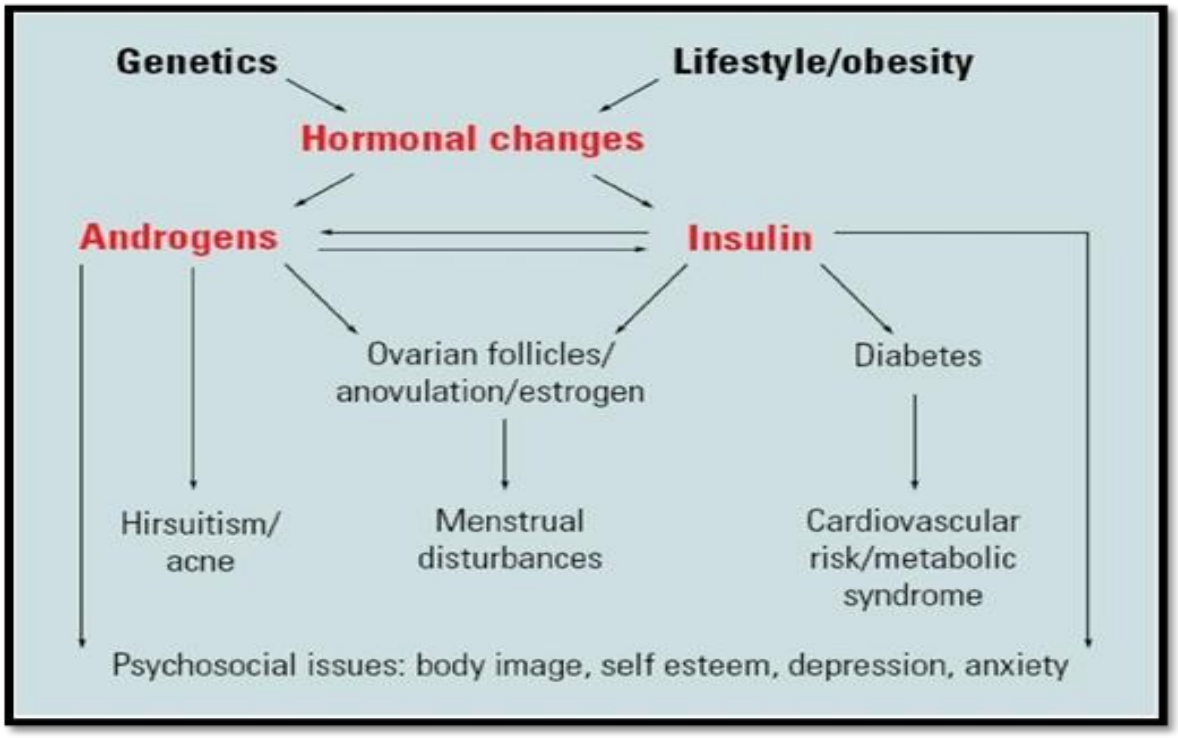


Figure (2.3): Scheme of aetiology and clinical features, including menstrual, cardiovascular/metabolic and psychosocial issues of a polycystic ovarian syndrome (Teede *et al*., 2010)

2.2.3. Prevalence of PCOS globally and locally

Polycystic ovary syndrome (PCOS) is a multifactorial disorder that has significant metabolic, reproductive and psychological consequences (Teede *et al*., 2010)It is a major public health concern affecting 6-10% of reproductive-aged women worldwide (Bozdag *et al*., 2016)The intricacy and difficulties of the existing diagnostic criteria must be recognized to determine the prevalence of PCOS in subgroup populations. There are three sets of diagnostic criteria used: those established by the National Institutes of Health (NIH) international conference on PCOS in 1990, the European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine (ESHRE/ASRM) in 2003 (referred to as the Rotterdam criteria), and the Androgen Excess Society and PCOS Society (AE-PCOS) in 2006 (Garad *et al*., 2011). To assess the presence or absence of PCOS, each set of criteria has slightly distinct

clinical, biochemical, and image-based results (Okoroh *et al.*, 2012). Changes in diagnostic criteria have a significant impact on the prevalence of PCOS. Prevalence rates have been recorded as low as 1.6 percent using all three criteria (Okoroh *et al.*, 2012) and as high as 18 percent using the Rotterdam criterion (Lim *et al.*, 2013) in similar populations. According to Futterweit's statistics, 50-75 percent of women with PCOS in the world are ignorant that they have the condition which may affect the prevalence statically analysis (Futterweit, 1999). Furthermore, various factors influence PCOS prevalence, including age, hormonal profile, lifestyle, smoking, physical activity, race, and geographical distribution, which vary from one population to another (Motlagh Asghari *et al.*, 2022)

In 2017, 1.55 million (95%) incident cases of PCOS among women of reproductive age (15–49 years) were reported globally, representing an increase of 4.47% (2.86–6.37%) from 2007 to 2017. The global age-standardized incidence rate of PCOS among women of reproductive age was 82.44 (64.65–100.24) per 100 000 population in 2017, which represents an increase of 1.45% (1.43–1.47%) from 2007 to 2017 (Liu *et al.*, 2021).

In Iraq, there are no accurate studies on the prevalence of PCOS, only one local study that was carried out in the north of Iraq, found that the prevalence of PCOS was 6.11% in the gynaecology out-patient visits and 35.39% among infertile women observed among infertile women attending In vitro fertilization (IVF) centre using the Rotterdam 2003 criteria for diagnosis (Hussein & Alalaf, 2013).

2.2.4. Pathogenesis of PCOS

polycystic ovary syndrome (PCOS) is considered a heterogeneous collection of signs and symptoms that form a range of a disorder with a mild presentation in some but a severe disturbance of reproductive, endocrine and metabolic function in others. PCOS pathophysiology appears to be multifactorial and polygenic, and many of theories were proposed to explain the pathogenesis of PCOS (Soni *et al.*, 2018):

First; A change in gonadotropin-releasing hormone secretion results in increased luteinizing hormone (LH) secretion is characteristic of the hallmark of PCOS. LH is secreted in a pulsing routine. PCOS women have an increase in the LH pulse frequency and capacity, resulting in increased 24-hour secretion. This increase in LH secretion is thought to occur as a result of the increased frequency of hypothalamic gonadotropin-releasing hormone (GnRH) pulses which leads to an increase in androgen production by the theca cells within the ovary(Patel *et al.*, 2003)

Second; A defect in androgen synthesis due to an increase in ovarian androgen production**.** The increase in LH with hyperinsulinemia leads to an increase in androgen production by ovarian theca cells (Tsilchorozidou *et al., 2004)*. Moreover, the most likely primary factor leading to an increase in testosterone secretion in PCOS is an increase in ovarian enzymatic activity involved in the synthesis of testosterone precursors (Hill, 2003)

Third; An alteration in insulin secretion and insulin action results in hyperinsulinemia and insulin resistance (IR) which is defined as (reduced glucose response to a given amount of insulin), and appears to be the common pathway of disease amongst women with PCOS. Increasing in the circulating levels of insulin has a direct effect on the ovaries by releasing other factors such as growth factor 1 (IGF-1) from the liver which employs an effect on the ovary. Moreover higher levels of insulin and IGF-1 in the

ovary leading to the release of higher levels of testosterone. All of these hormones (including insulin, IGF-1 and testosterone) prevent the growth of ovarian follicles during the ovulation, leading to an accumulation of small ovarian follicles less than 10 mm diameter that do not progress through the ovulation(Johnson, 2014)

In PCOS, Hyperinsulinemia, hyperandrogenemia and altered intraovarian paracrine signaling can disrupt ovarian follicle growth, The consequent follicular arrest in PCOS is accompanied by anovulatory, menstrual irregularity ,subfertility and the accumulation of small antral follicles within the ovary, giving it a polycystic morphology (Goodarzi *et al.*, 2011) (Figure 2.4).

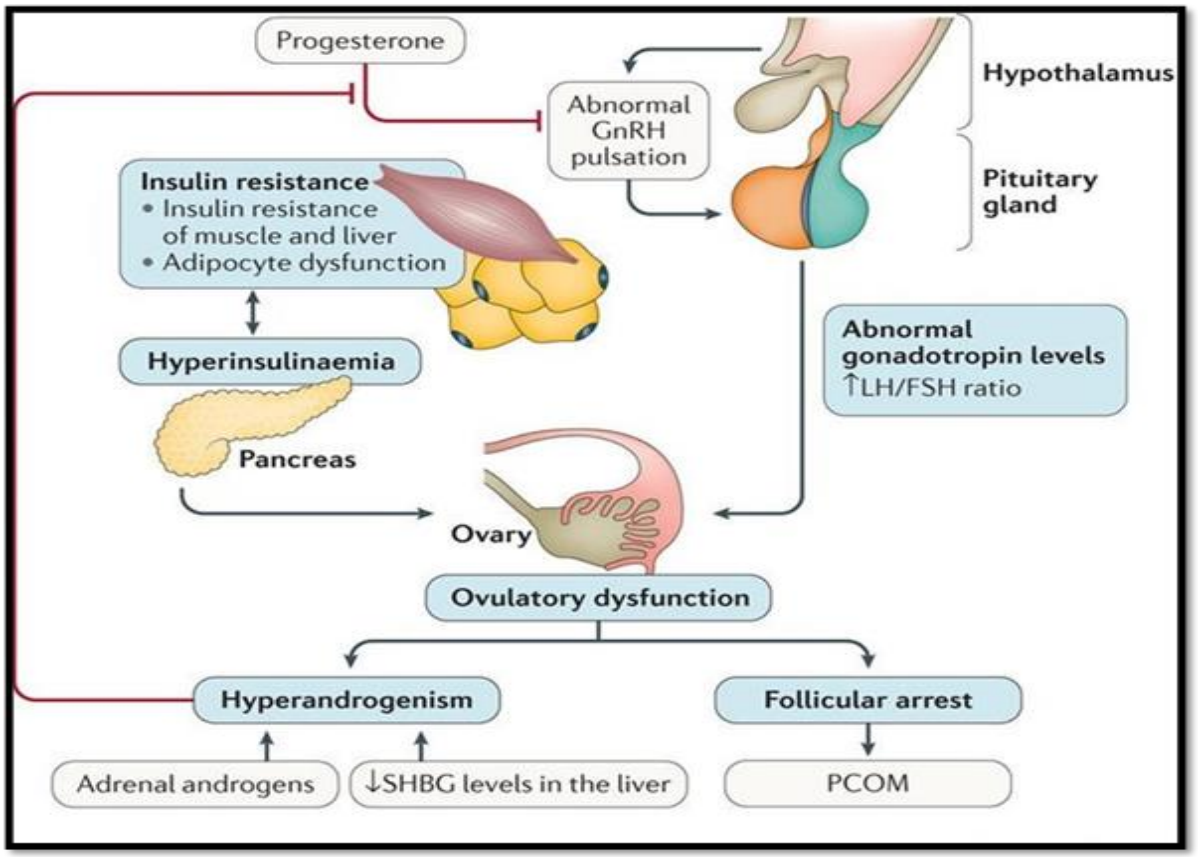


Figure (2.4): The pathophysiology of polycystic ovary syndrome (Taffy *et al*.,2019)

2.3. Hormones associated with PCOS

2.3.1. Luteinizing Hormone (LH)

The pituitary gland's anterior lobe is stimulated by (GnRH) to produce LH. At midcycle, there is an LH surge that induces an increase in maturation-promoting factor concentrations, causing oocytes to complete meiosis I and begin meiosis II; it also increases progesterone production by follicular stromal cells (luteinization); and it causes follicular rupture and ovulation (Nedresky & Singh, 2021).

In conjunction with Follicle Stimulating Hormone (FSH), luteinizing hormone promotes follicular growth and ovulation. As a result of the complimentary activity of FSH and LH, proper follicular expansion occurs (Raju *et al.*, 2013). In addition, LH operates in the ovary after ovulation to maintain the corpus luteum's release of predominantly progesterone to prepare the uterus for future implantation (Palomba *et al.*, 2015).

In PCOS women, abnormality of the hypothalamic-pituitary-ovarian axis has been compulsory in the pathophysiology of polycystic ovarian disease. disorder in the secretion pattern of the gonadotrophin-releasing hormone (GnRH) results in a relative increase in LH to FSH release (Saadia, 2020). Usually, in healthy women, the ratio between LH and FSH lies between 1 and 2. In polycystic ovary disease women, this ratio becomes reversed, and it might reach as high as 2 or 3 ; as a result of raised LH/FSH ratio, ovulation does not occur in polycystic ovary disease patients (Johansson & Stener-Victorin, 2013). Many studies have been reported on LH elevation in PCOS patients as (Deswal *et al*., 2019)and found a significant change in the level of LH hormone in PCOS patients. Moreover, oxidative stress in PCOS patients can play a vital role in LH,

FSH secretion and hence the evolution process in which oxidative stress causes anxiety, depression and mitochondrial dysfunction. The Hypothalamus pituitary axis is stimulated by stress, triggering the excess secretion of cortisol from the adrenals, releasing the inflammatory markers with increased lipid peroxidation and oxidative stress (OS).cortisol secretion suppresses the gonadotropin-releasing hormone (GnRH), which then decreases the release of luteinizing hormone (LH) and follicular stimulating hormone (FSH). Failure to stimulate ovaries to secrete estradiol and with mitochondrial dysfunction, oocyte maturation failure occurs in PCOS (Alam *et al.*, 2019).(Figure 2.5)

Figure(2.5): Effect of oxidative stress on LH,FSH secretion, and oocyte maturation (Alam *et al.*, 2019)

2.3.2. Follicle-Stimulating Hormone (FSH)

The hypothalamus produces gonadotropin-releasing hormone (GnRH), which acts on pituitary gland cells in the anterior lobe (adenohypophysis) to secrete gonadotropins. These hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), stimulate and regulate ovarian cyclic changes. FSH stimulates the growth of approximately 15 to 20 primary-stage (preantral) follicles at the start of each ovarian cycle. Although the presence of FSH is not required to promote the development of primordial follicles to the primary follicle stage, these primary follicles die and become atretic if it is not present (Marques *et al.*, 2022).

LH and FSH are the hormones that encourage ovulation and are secreted by the pituitary gland in the brain. Most women have equal amounts of LH. and FSH during the early part of their cycle. However, there is an LH. Surge in which the LH amount increases 24 hours before ovulation occurs. Once the ovary releases the egg, the LH levels go back down. While many women with PCOS have LH level is often two or three times that of the FSH level. This situation is called an elevated LH to FSH ratio or a ratio of 3:1. This change in the LH to FSH ratio is enough to disrupt ovulation. While this used to be considered an essential aspect in diagnosing PCOS (Raju *et al*., 2013)

Regulation of oxidative stress (OS) is important to prevent damage to female reproductive physiology. While normal OS levels may have a regulatory role, high OS levels may negatively affect vital processes such as folliculogenesis.(Martín-Ramírez *et al.*, 2021). FSH is a glycoprotein released by the pituitary gland that plays an important role in encouraging follicle development and regulating ovarian function during folliculogenesis (Huang *et al.*, 2019). FSH is a key survival factor for antral follicles, and it has been suggested that it can boost granulosa cell (GC)

tolerance to oxidative stress during follicular apoptosis(Shen *et al.*, 2014). FSH has been proposed to play a unique role in protecting the ovary from oxidative damage (Shen *et al.*, 2017). fertility diseases like endometriosis and polycystic ovary syndrome, contribute to increased OS levels and enhance the impact of OS on female reproduction (A. Agarwal *et al.*, 2012). Previous studies in women who have fertility treatments showed a relationship between higher oxidative stress biomarker levels and lower oocyte fertilization potential (Tamur*a et al.*, 2008).

2.4. Treatment of PCOS

Management of PCOS was based on the type and extent of the disorders and if there is a need for pregnancy, the goals of the therapeutic program include improvement in menstrual cycles, reduce circulating androgens, reduce insulin resistance, prevent metabolic complications, decrease cardiovascular risk, weight loss achievement, improving the response to ovulation induction therapy, treatment of infertility and prevent endometrial carcinoma (Legro, 2015)

The First line in the treatment of PCOS is the non-medication approach, which includes lifestyle modification through diet and exercise. There is a significant correlation between hyperinsulinemia and obesity in PCOS, which is mostly associated with a decrease in ovarian function. Weight loss may help to improve IR by decreasing its harmful effect on the hormonal disorder, ovulation and menstrual regularity (Saleem & Rizvi, 2017)

Medical treatment of PCOS can be divided into two components depending on the type of case, one of which is considered as acute case and need control of irregular menses and treatment of hirsutism, other is more chronic which needs the management of infertility (Artini *et al.*, 2010). Irregular menses can be controlled by oral contraceptives, which are effective in normalizing menstrual cycles and decreasing bioavailable

testosterone levels to minimize hirsutism (Diamanti-Kandarakis et al., 2003).

Typically, treatment of PCOS focuses on insulin-sensitizing agents which including metformin and thiazolidinediones such as troglitazone, which aid the reduction of insulin resistance and subsequent hyperinsulinaemia which is used to assist insulin sensitivity and reduction of androgen levels(Arlt *et al.*, 2001). This has also been seen with pioglitazone and rosiglitazone but is only effective at supra-physiological concentrations. The androgen-lowering potential of those TZDs in current clinical use may therefore be effective through a more indirect mechanism (Balen, 1993).

2.4.1. Metformin

Metformin or Glucophage (commercial name) has a chemical structure ($C_4H_{11}N_5$) as shown in figure (2.6). It is the first-line drug of choice for the treatment of Type 2 diabetes mellitus (T2DM), particularly in overweight and obese people(Nasri & Rafieian-Kopaei, 2014). Metformin acts by refining the sensitivity of peripheral tissues to insulin which results in a reduction of circulating insulin levels and increases glucose uptake by peripheral tissues leading to a decrease in insulin resistance (IR)(Bailey, 1996; Dmitri *et al.*, 2002).

Metformin has been seen to reduce hyperandrogenemia and the direct mechanisms behind this, it is indirectly linked to both the reductions in circulatory insulin and the possible inhibition of steroidogenic enzymes. Research has shown that metformin stimulates adenosine monophosphate-activated protein kinase (AMP-K) a major cellular regulator of lipid and glucose metabolism. In doing so glucose uptake is increased (Zhou *et al.*, 2001). Additionally, metformin has been seen to alleviate glucose toxicity aiding glycemic control and lipid regulation (DeFronzo *et al.*, 1995). In

some cases, metformin can inhibit the effects of CYPl7 and may therefore explain reductions in androgen levels (La Marca *et al*., 2004) however contradictory research in human studies has shown that no effect is seen suggesting any reductions in androgen is secondary to the effects metformin has on insulin levels (Arlt *et al.*, 2001).

Side effects of metformin included gastrointestinal symptoms such as nausea, diarrhoea, flatulence, bloating, anorexia, metallic taste, and stomach pain. These symptoms can be decreased in patients to varying degrees by gradually administering metformin and titrating the dose increase based on the severity of symptoms(Nestler, 2008). Velazquez and colleagues reported in an observational study a significant improvement in menstrual regularity and reduction in circulating androgen levels (Velazquez *et al.,* 1994)as well as a significant reduction in body weight, which confounded their findings.

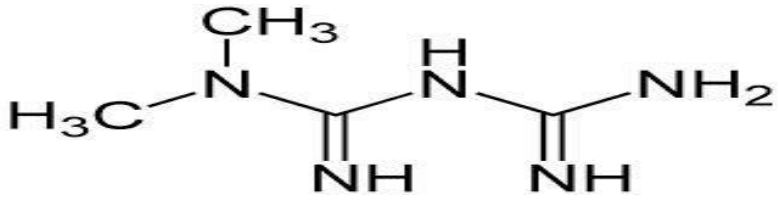


Figure (2.6): Metformin structure (Rizvi *et al.*, 2015)

2.5. Oxidative stress

Oxidative stress (OS) can be defined as an imbalance between increased levels of reactive oxygen species (ROS) and a low activity of antioxidant mechanisms. They are normally generated as by-products of oxygen metabolism, mainly by mitochondria, during physiological and pathological conditions in which $O_2^{\bullet -}$ can be formed by cellular respiration. Also, they are required for cell function, including the production of energy by the mitochondria. An increase in oxidative stress can induce damage to the cellular structure and possibly destroy tissues (Preiser, 2012).

ROS are the active form of oxygen that is needed for several cellular functions and in response to different stimulation, the diatomic oxygen molecule (O2) does not react spontaneously with other molecules as it contains two unpaired electrons. The reaction of oxygen with organic molecules was achieved either by oxidation or reduction(Magder, 2006), and affected several of the cellular compounds, which can lead to:

1. DNA modifications by degradation of bases, single or double-stranded DNA breaks pyrimidine, purine, or sugar-bound modifications, mutations, deletions, translocations and cross-linking with proteins which highly related to carcinogenesis, ageing, and neurodegenerative, cardiovascular, and autoimmune diseases. Also affects Promoter regions by attacking the transcription factor–binding sites and can modify the binding of transcription factors and thus change the expression of related genes (Ghosh & Mitchell, 1999)

2. lipid peroxidation disturbs the membrane lipid bilayer arrangement which leads to the inactivation of membrane-bound receptors and enzymes as well as increased tissue permeability(Girotti, 1998). Products of lipid peroxidation, such as MDA and unsaturated aldehydes, can inactivate several cellular proteins by forming protein cross-linkages that cause depletion of intracellular GSH and induces peroxide production (Keller *et al.*, 1997).

3. Protein denaturation by fragmentation of the peptide chain, alteration in electrical charge of proteins, and oxidation of specific amino acids that lead to increased exposure to proteolysis and degradation(Kelly & Mudway, 2003), Enzymes that have metals on their active sites are mainly more sensitive to metal oxidation and modification of enzymes which leads to inhibit their activities(Stadtman, 1990). protein modification has been

directly associated with the development of human diseases, such as Alzheimer's, autoimmune diseases, Crohn's disease, kidney diseases, and Parkinson's disease (López-Alarcón *et al.*, 2014)

Oxidative stress includes exogenous factors, such as cigarette smoke, ozone exposure, ion radiation, alcohol consumption, strenuous physical activity, poor diet, and Endogenous factors like the oxidative burst from activated macrophages, producers from ROS NADPH oxidase in cell membranes, mitochondria, peroxisomes (Birben *et al.*, 2012).

The imbalance between pro-oxidants and antioxidants and oxidative stress can lead to several reproductive diseases such as polycystic ovary syndrome (PCOS), endometriosis, and unexplained infertility. Endometriosis, polycystic ovarian syndrome (PCOS), and unexplained infertility can be caused by an imbalance of pro-oxidants and antioxidants, as well as oxidative stress(Tesarik, 2021). In reaction to oxidative stress, pregnancy problems such as spontaneous abortion, recurrent pregnancy loss, and hypertension can develop. Excessive body weight and lifestyle factors such as cigarette smoking, alcohol use, and recreational drug use have been found to induce excess free radical generation, which may impact fertility. Environmental toxins are causing oxidative states, which may contribute to female infertility (Ashok Agarwal *et al.*, 2012).

Oxidative stress has been associated with PCOS. According to meta-analysis research, circulating markers of oxidative stress are abnormal in women with PCOS, suggesting that oxidative stress plays a role in the pathogenesis of PCOS. Oxidative stress has a role in PCOS by altering steroidogenesis in the ovaries, which leads to increased androgen levels, disrupted follicular development, and infertility (Sulaiman *et al.*, 2018). Furthermore, obesity, insulin resistance, and cardiovascular risks have been linked to oxidative stress in PCOS women (Lorenz & Wild, 2007)

2.5.1. Free radical

Free radicals are the products of normal cellular metabolism, which can be defined as an atom or molecule, that have one or more unpaired electrons in the outer orbit. The odd number of electrons of a free radical makes it unstable, short-lived and highly reactive, therefore, it can catch electrons from other compounds to reach stability. As a result, these molecules lose their electrons and become free radicals, triggering a chain reaction cascade that eventually damages the living cell(Phaniendra *et al*., 2015). ROS and RNS play a vital role as useful and toxic compounds to the living system. At the moderate level, they are involved in various physiological functions such as immune function, cellular signalling pathways, mitogenic response and in redox regulation (Valko *et al.,* 2007), While at higher concentrations generate oxidative stress and cause potential damage to the biomolecules, as oxidative stress is developed when there is an excess production of ROS and RNS or in deficiency of enzymatic and non-enzymatic antioxidants (Phaniendra *et al*., 2015).

ROS and RNS collectively constitute both the free radicals and non-radical reactive species:

The radicals include Superoxide ($O^{\cdot-}_2$), Oxygen radical ($O^{\cdot\cdot}_2$), Hydroxyl ($OH^{\cdot}$), Alkoxyradical ($RO^{\cdot}$), Peroxyl radical ($ROO^{\cdot}$), Nitric oxide ($NO^{\cdot}$) and nitrogen dioxide ($NO^{\cdot}_2$)(Hilali *et al*., 2013).

The non-radical species include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), singlet oxygen ($^1O_2$), nitrous acid (HNO_2), nitrosyl cation (NO^+), nitroxyl anion (NO^-), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), nitronium cation (NO_2^+), organic peroxides (ROOH), aldehydes (HCOR) and peroxynitrite (ONOOH)(Kohen & Nyska, 2002). These non-radical species are not free radicals but can simply lead to free radical reactions in living organisms(Genestra, 2007).

2.5.2. ROS in normal physiology

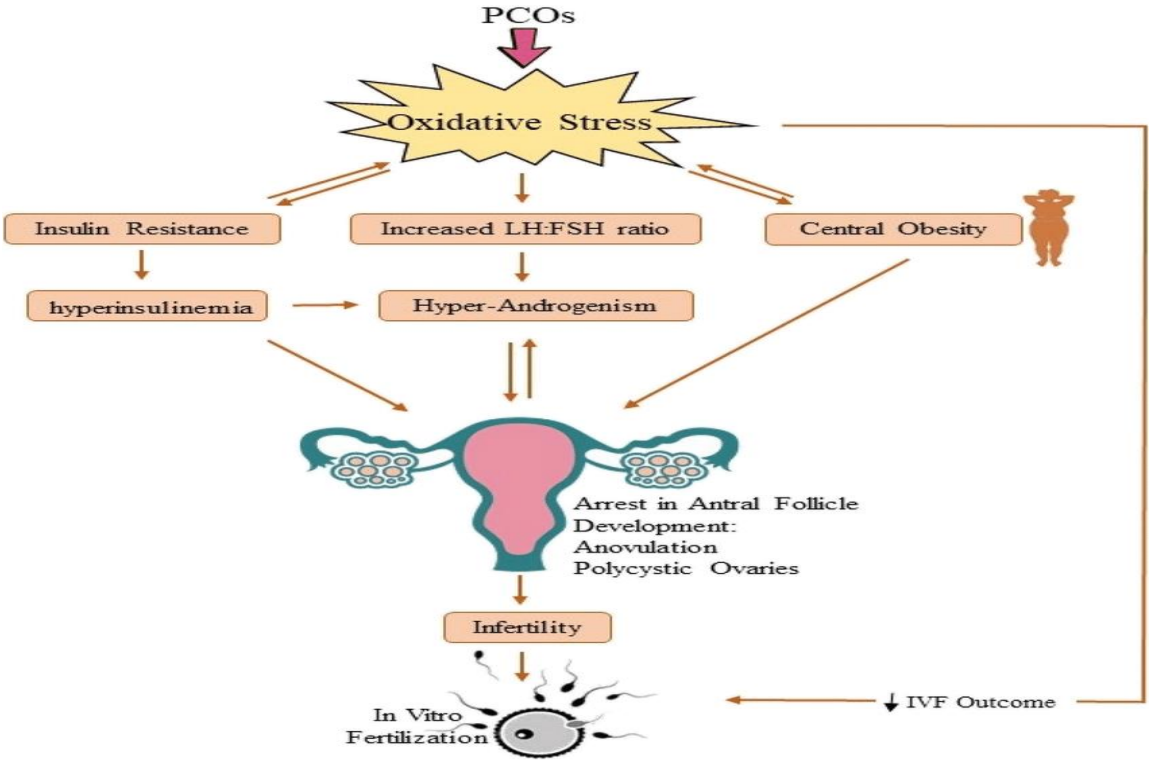
Normally, a low concentration of ROS is necessary for normal physiological functions such as gene expression ،cellular growth, and defence against infection.ROS serve as secondary messengers in many developmental stages of prenatal and embryonic growth in mammals (Kunwar & Priyadarsini, 2011). ROS participate in the biosynthesis of molecules such as prostaglandin that accelerate developmental processes and thyroxin synthesis that, regulated by H_2O_2 concentration, catalyze the binding of iodine atoms to thyroglobulin (Shulaev & Oliver, 2006). Finally, ROS are involved in the immune system via inducing the proliferation of T cells, which were admitted to generating ROS to destroy bacterial cells for engulfing by the phagocyte.

2.6. Oxidative stress and PCOS

Polycystic ovarian syndrome (PCOS) is a highly complex and heterogeneous endocrine metabolic disorder, characterized by oligo-ovulation or anovulation, biochemical or clinical hyperandrogenism, presence of polycystic ovaries and associated with metabolic disorders, including insulin resistance, obesity and diabetes (Rotterdam.2004). Several studies have found that when the oxidative state is evaluated using circulating markers such as superoxide dismutase (SOD) and catalase, the OS level in PCOS patients is much higher than in the normal community. However, obesity, insulin resistance, hyperandrogenemia, and chronic inflammation are all found to be strongly linked with OS level (Murri *et al*., 2013)

The profound factors in PCOS that increase oxidative stress and obesity, IR, and hyperglycemia; however, non-obese PCOS women without IR are also reported to increasing oxidant status (Verit & Erel, 2008), suggesting that other factors may also contribute to inducing the

production of ROS in these women, Recent studies found that the elevation of circulating androgens is associated with high oxidative stress in women with or without PCOS (Gonzalez *et al.*, 2012). Oxidative stress appears to be involved in PCOS by causing altered steroidogenesis in the ovaries, which subsequently contributes to increased androgen levels, disturbing follicular development, and infertility (Sulaiman *et al.*, 2018). Elevated levels of ROS and reduced antioxidant capacity are closely related to reduced oocyte maturation and low embryo quality. As these molecules may reduce oocyte quality by changing the equilibrium of follicular fluid in the follicular microenvironment (Gharaei et al., 2021). (figure 2.7)



Figure(2.7):Relationship between oxidative stress and PCOS complications (Gharaei *et al.*, 2021).

2.7. Antioxidant system

Antioxidants are structurally diverse groups of small organic molecules and enzymes that form complex systems of overlapping activities. Act synergistically to improve cellular defence and counter oxidative stress

caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). Antioxidants are classified into enzymatic and non-enzymatic antioxidants. The non-enzymatic antioxidants intercept and terminate free radical chain reactions. Such as vitamins E, A, and C, flavonoids, carotenoids, glutathione, plant polyphenols, uric acid, curcumin, melatonin, and bilirubin(Agarwal *et al.,* 2008). Vitamin C and Vitamin E were included in this study:

1. Vitamin C or ascorbic acid is the major non-enzymatic water-soluble antioxidant in the biological system, which acts as a cofactor in several metabolic reactions required for a wide range of biological functions. Humans lost the capability to synthesize ascorbic acid due to a defect in L-gulono-1,4-lactone oxidase that catalyzes the transformation of L-glucono lactone into ascorbic acid, therefore humans depend on the diet as a source for vitamin C to prevent the vitamin C deficiency. Vitamin C can be attributed to several biological functions as a cofactor for a numeral of enzymes like hydroxylases that are involved in collagen synthesis and as a water-soluble antioxidant (Traber & Stevens, 2011)

Vitamin C generally works as an antioxidant by directly reacting with ROS and has a vital role in defences against oxidative stress and preventing oxidative damage to important biological macromolecules such as DNA, lipids, and proteins and reduces redox-active transition metal ions in the active site of specific biosynthetic enzymes(Carr & Frei, 1999). The oxidized vitamin C, dehydroascorbic acid (DHA), stimulates the antioxidant defences of cells(Puskas *et al*., 2000).

Vitamin C plays a vital role in regulating the menstrual cycle and ovarian function in which ascorbic acid excretion declines directly before ovulation, then increases again after the temperature rises post-ovulation. Ascorbic acid levels are stimulatory to progesterone hormones and have

been found in high concentrations in the corpus luteum (Varshney & Kale, 1990). Vitamin C in the ovaries is responsible for collagen synthesis, which is required for follicle and corpus luteum growth. Problems in this function may contribute to the progress of ovarian cysts and PCOS(Chen *et al.*, 2005)

2. Vitamin E, a lipid-soluble substance known as tocopherol, was found by Evans and Bishop in 1922 (Evans & Bishop, 1922). Vitamin E can antagonize the oxidative stress caused by the oxygen free radicals and antioxidant imbalance by rapidly transferring its phenolic hydrogen atom to neutralize free radicals and regulate the normal physiological function of the reproductive system. vitamin E can reduce the oxidative stress reaction that may have a harmful effect on the number and quality of oocytes(Tarín *et al*., 2002). A lack of vitamin E can cause female infertility, eclampsia, miscarriage, premature delivery, fetal intrauterine growth restriction, and other pregnancy-related diseases(Hubalek *et al.*, 2014)

While enzymatic antioxidants function by converting oxidized metabolic products in a multi-step process to hydrogen peroxide (H_2O_2) and then to water using cofactors such as iron, zinc, copper, and manganese superoxide dismutase(SOD), catalase, and GPx are examples of enzymatic antioxidants. (Agarwal *et al.,* 2008)Antioxidants prevent and limit the adverse effects of oxygen radicals and have essential roles in the female reproductive system as well as in the pathogenesis of female infertility (Kusçu NK and Var A .2009). Changes in the antioxidant concentrations in serum and peritoneal fluid have been studied in idiopathic infertility, tubal infertility, and endometriosis patients. Investigations of antioxidant concentrations in PCOS patients are promising and numerous studies have measured antioxidant markers to correlate ROS and PCOS and the various clinical indicators of metabolic syndrome, including diabetes, obesity, and cardiovascular diseases (Jozwik *et al.,* 1999).

Oxygen species are critical participants in several diseases such as virus infections which cause airway epithelial inflammation, progression to cancer, neurodegenerative processes that include cell death, motor neuron diseases and axonal injury, and both infarction and brain oedema (Uttara *et al.*, 2009). Tissues were protected from this oxidative injury by expressing stress-response genes and genes encoding antioxidant enzymes (MatÉs *et al.*, 1999).

2.7.1. Superoxide dismutase enzyme (SOD enzyme)

In 1967 biochemists, Irwin Fridovitch of Duke University and Joe McCord discovered the antioxidant enzyme SOD, which provides an essential cellular defence against free radical damage. This discovery prompted medical scientists to begin to look seriously at free radicals (Pillai & Pillai, 2002). Antioxidant enzymes include superoxide dismutase, catalase, glutathione peroxidase, and recently appreciated thioredoxin. All of the enzymes play vital roles in the modulation of oxidative stress; the primary role in the superoxide anion radical metabolism is employed by superoxide dismutase, which catalyzes the dismutation of superoxide to hydrogen peroxide (Faraci & Didion, 2004).

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

There are three major forms of superoxide dismutases, MnSOD enzyme that is present in mitochondria, Cu/znSOD enzyme in the cytoplasm, and extracellular SOD (EC-SOD) enzyme, which is secreted by vascular cells into the extracellular matrix.

Superoxide dismutase SOD (EC 1.15.1.1) is an enzyme that catalyzes the dismutation of the superoxide (O^-_2) radical into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Superoxide radical is created as a by-product of oxygen metabolism and, if not regulated, can cause many types

of cell damage (Hayyan *et al.*, 2016); Hydrogen peroxide is then degraded by other enzymes such as catalase. Thus, SOD is an essential antioxidant defence in almost all living cells exposed to oxygen.

2.7.1.1. (Cu\Zn) SOD enzyme

Cu/Zn SOD is a metalloprotein with a molecular mass of approximately 32 000 kDa and has two protein subunits, each containing a catalytically active copper and zinc atom; each subunit is composed of eight antiparallel β strands that form a flattened cylinder, plus three external loops, with the active site held between the barrel and surface loops(figure2.8). The two subunits are tightly joined back-to-back by hydrophobic and electrostatic interactions. The active sites Cu (II) and Zn(II) lie 6.3 Å apart at the bottom of this long channel; the Zn is hidden, while the Cu is accessible for solvent. The side chain of His61 forms a bridge between Cu and Zn and is coplanar with them (Tainer et al., 1982).SOD1 is found in mammalian cells and is expressed at relatively high levels in blood vessels; the activity of SOD1 accounts for 50–80% of total SOD activity (Horiuchi *et al.*, 2004). In physiological conditions, the superoxide dismutase and the non-enzymatic ROS scavengers such as vitamins E, A, and C maintain a steady state between oxidant and antioxidant systems (Russo *et al.,* 2011).This enzyme requires both Cu and Zn to function biologically, and the lack of Cu causes complete inactivation, which frequently results in the development of diseases in humans(BROWN & Besinger, 1998) . Cu/Zn SOD has significant therapeutic potential and physiological importance. This enzyme's function has been studied in relation to a number of distinct red blood cell (RBC) diseases, such as cystic fibrosis, iron deficiency anemia, oxidative hemolytic anemia, thalassemia, sickle cell anemia, and muscular dystrophy(Mavelli *et al.,* 1984). This enzyme has also been linked in recent research to amyotrophic lateral sclerosis, malign breast

disease, steroid-sensitive nephrotic syndrome, and dengue fever(Stieber *et al.*, 2000). SOD activity is markedly elevated in rheumatoid arthritis, ischemia damage, and cancer, pointing to superoxide-related pathophysiology and the involvement of SOD (Noor *et al.*, 2002).

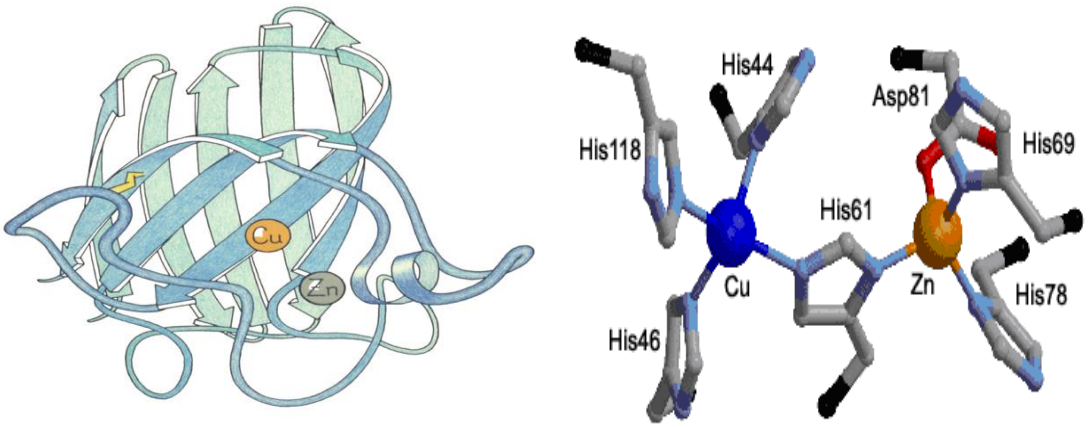


Figure (2.8): The structure of Cu/Zn SOD and the active site (Josko, Osredkar ;and Natasa, Sustar,2011) (Tainer JA *et al.*,1982)

2.7.1.1.1 Genetic structures and organization of the *SOD1* gene

The human *sod1* gene is localized on chromosome 21q22; the latest information on *sod* genes sequences is generated from initial sequencing data and comparative analysis of whole genomic studies among different species, which revealed that the *sod1* gene consists of five exons interrupted by four introns, the variation of intron size found in different studies may be associated with gene polymorphisms in different human tissues and cell lines. The *sod1* promoter has a high GC-rich region, as well as the TATA box and CCAAT box (Levanon *et al.*, 1985).

Oxidative stress is one of the most studied cellular conditions due to its relation to several diseases. When the cells are under oxidative conditions, the antioxidant defences are activated. The levels of expression of the genes related to antioxidant protection are regulated by transcriptional and posttranscriptional mechanisms (Lu *et al.*, 2007). Human SOD1 is a

protein for cellular antioxidant defence. Some studies have focused on better understanding the structure, mechanism, and regulation of human SOD1 mRNA levels under normal and oxidative conditions (Milani *et al.*, 2011).

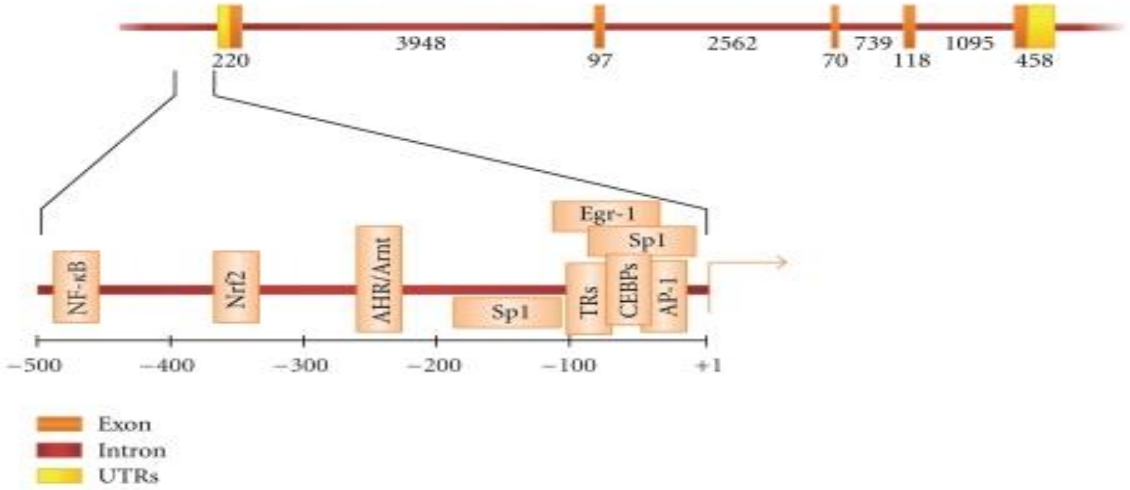


Figure (2.9): Genomic organization of human *SOD1* gene. exons and introns size in base pairs are shown in relationship with each fragment. The 5′ flanking regions are expanded, and the transcription factors, which react with the corresponding DNA regulatory elements, are shown at the bottom. The transcription's initial site is depicted as an arrow at position +1 (Milani *et al.*, 2011)

2.7.1.2. MnSOD enzyme (SOD2)

Manganese superoxide dismutase (MnSOD), Superoxide dismutase 2, and mitochondrial SOD are present in mitochondria that is mediated ROS generated by the partial reduction of O_2. MnSOD act as the first line of defence against reactive oxygen species by catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2).

$$Mn^{3+}+O_2^{\bullet-} \leftrightarrow Mn^{2+}+O_2$$

$$Mn^{2+}+O_2^{\bullet-}+2H^{+} \leftrightarrow Mn^{3+}+H_2O_2$$

Alteration in the function or expression of MnSOD can have significant consequences on mitochondrial function and the general health of cells due to oxidative damage in various mitochondria-localized metabolic processes, leading to the development of different diseases (Hainaut &

Milner, 1993; Miao & Clair, 2009). The *SOD2* gene encodes the MnSOD enzyme on chromosome six which forms a homotetramer and binds one manganese ion per subunit as a cofactor (Perry *et al.*, 2009). The metal is organized by the His26, His74, His163, and Asp159 residue and an oxygen-containing molecule, that can be either water or a hydroxide(Bonetta, 2018). The amino acids layer in the active site of the enzyme is essential to perform the dismutation reaction. The amino acids crucial in the enzyme catalysis mechanism include His30, Tyr34, Phe77, Trp78, Trp123, Gln143, Trp161 and Glu162 from the adjacent subunit(Azadmanesh *et al.*, 2017).(Figure 2.10)

Superoxide anions have proinflammatory roles, causing lipid peroxidation and oxidation, DNA damage, peroxynitrite ion formation and recruitment of neutrophils to sites of inflammation (Droy-Lefaix *et al.*, 1991). Moreover, the SOD2 enzyme is an important enzyme in apoptotic signalling and oxidative stress and plays a vital role in the mitochondrial death pathway, which is required for successful embryonic development and the maintenance of normal tissue homeostasis. (Danial & Korsmeyer, 2004). Numerous researchers have revealed that oxidative stress is significantly increased in women with PCOS (Mohammadi, 2019), and there is an association between Mn superoxide dismutase (SOD2) and PCOS (Azadmanesh & Borgstahl, 2018)

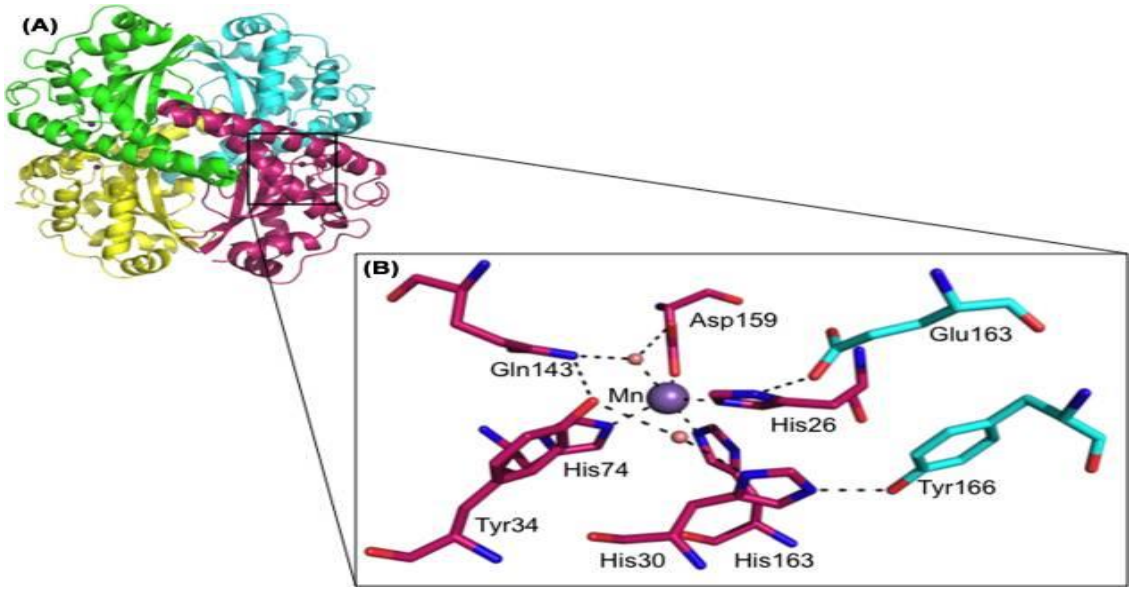


Figure (2.10) : The quaternary and active site structure of human MnSOD.(A) Human MnSOD contains four subunits to form the tetramer. (B) The active site of the human MnSOD enzyme depicts the hydrogen-bonding network from the direction of access to the substrate. (Bonetta, 2018).

2.7.1.2.1. Genetic structures and organization of the *sod2* gene

The human *sod2* is located on chromosome 6q25.3. Based on the molecular structure and organization of the human *sod2* gene, five exons interrupted by 4 introns have been identified. The basal promoter of the *sod2* gene lacks TATA and CAAT boxes but contains GC-rich motifs and numerous Sp1 as well as several AP-2 consensus sequences in its proximal promoter region(WAN et al., 1994).figure(2.11)

A number of studies was identified the association of *sod2* genetic polymorphisms with various diseases including diabetes and hypertension(Nakanishi *et al.,* 2008) Mutations detected in the *sod2* promoter region reveal the possibility for decreased expression of MnSOD in several human cancer cells (Xu *et al*., 1999).

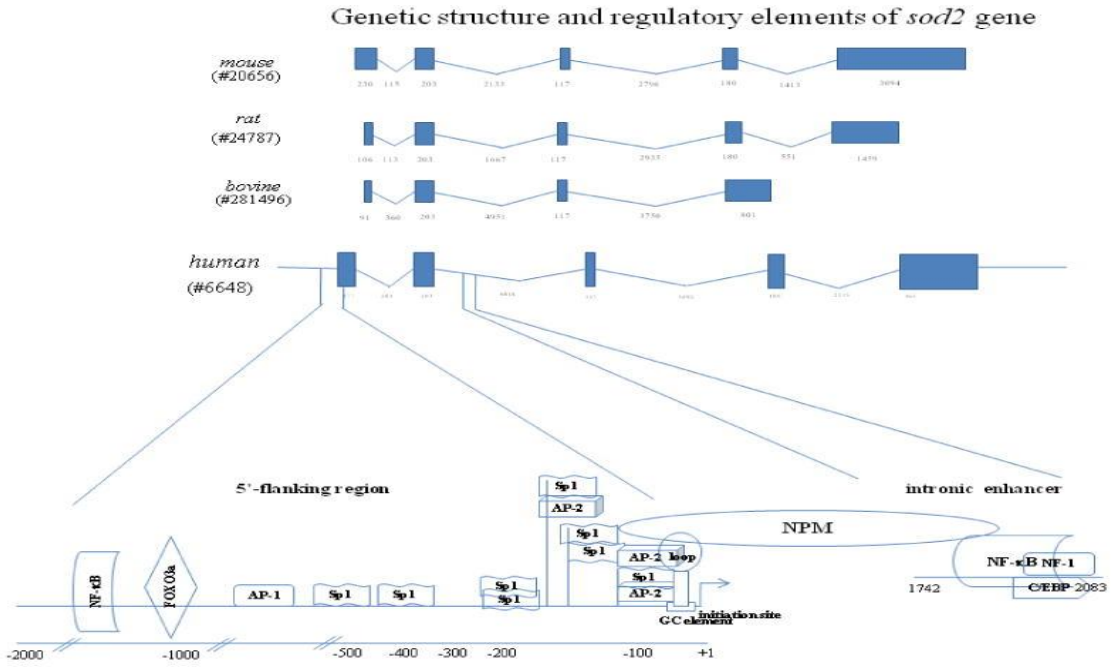


Figure (2.11): Organization of the *sod2* gene, The regulatory elements identified in the 5′ flanking regions and the second intron of the human *sod2* are expanded and shown in the lower part. Corresponding numbers with positive and negative numbers indicate their location relative to the start site, which is designated +1.(Miao & Clair, 2009)

2.7.2. Catalase enzyme

Catalase (EC 1.11.1.6) is one of the most important antioxidant enzymes which detoxify H_2O_2 (A harmful by-product of many normal metabolic processes) into oxygen and water, preventing cellular injury from reactive oxygen species (Wenten M *et al* 2009). Human catalase forms a tetramer that is composed of four subunits, each of which can be divided into four domains. The extensive core of each subunit is generated by an eight-stranded antiparallel β-barrel, A helical domain at one face of the β-barrel is composed of four C-terminal helices and four helices derived from residues between β4 and β5 (Putnam, 2000).

Catalase contains four porphyrin heme or iron groups which allow the enzyme to react with the hydrogen peroxide in which hydrogen peroxide

enters the active site and interacts with the amino acids asparagine and Histidine, causing hydrogen ions to transfer between the oxygen atoms, freeing the newly formed water molecule and Fe(IV)=O. then Fe(IV)=O reacts with a second hydrogen peroxide molecule to reform Fe(III)-E and produce water and oxygen(Karakus, 2020). This enzyme breaks down two hydrogen peroxide molecules into one molecule of oxygen and two molecules of water in a two-step reaction (Nandi *et al.*, 2019) as the following:

$$H_2O_2 + \text{Fe(III)-E} \rightarrow H_2O + \text{O=Fe(IV)-E(.+)}$$
$$H_2O_2 + \text{O=Fe(IV)-E(.+)} \rightarrow H_2O + \text{Fe(III)-E} + O_2$$

The distribution of catalase during different ovarian cycles is related to gonadotropin regulation. Gonadotropins such as FSH have an important function for follicular maturation, differentiation, and steroidogenesis (Fortune J. E.1995).catalase activity was significantly enhanced by gonadotropin stimulation in different mammals. Catalase and estradiol activities in ovarian granulosa cells in different follicle stages were related to FSH levels. Catalase activity increased after FSH stimulation, and the degree of this increase was greater in large follicles than in medium or small follicles; that may suggest a role of catalase in follicle selection and prevention of apoptosis(Parshad & Guraya, 1993).

2.8. Role of SOD genes in PCOS

SOD1 and *SOD2* genes are located on chromosomes 21q22.11 and 6q25.3, expressed in all human tissues (Polat & Şimşek, 2020). it accounts for approximately 50-80% of the total SOD activity and is considered an excellent device against oxidative stress (Weydert & Cullen, 2010).

Insulin resistance has a central role in PCOS. Signs of insulin resistance like hypertension, obesity, metabolic syndrome, nonalcoholic fatty liver, and sleep apnea (Vassilatou, 2014). Most importantly, waist

circumference, independent of body mass index, is responsible for an increase in the oxidation of LDL (Weinbrenner *et al*., 2006). Insulin resistance and hyperinsulinemia increase luteinizing hormone (LH) and the availability of circulating androgen and androgen production by the adrenal gland and ovary mainly by decreasing sex hormone binding globulin (SHBG).(Baptiste *et al.,* 2010). PCOS is associated with decreased antioxidant concentrations and is thus considered an oxidative state (Zuo *et a*l., 2016). The decrease in mitochondrial O_2 consumption, GSH levels, and ROS production explains the mitochondrial dysfunction in PCOS patients(Victor *et al.,* 2011). Increased levels of ROS produced by mononuclear cells during physiological hyperglycemia trigger the release of TNF-alpha and an increase in the inflammatory transcription factor NF-kappa B. TNF-alpha levels, a recognized modulator of insulin resistance, consequently rise even more. As oxidative stress increases, an inflammatory milieu is created, which worsens insulin resistance and causes hyperandrogenism, inhibiting the dominant follicle's growth and preventing apoptosis of small follicles, leading to cyst formation in the ovaries. Hormonal imbalances in females with PCOS are decreased follicle-stimulating hormone (FSH) and FSH/LH ratio, an increase in the level of LH, fasting insulin, estrogen, free testosterone, and a mild increase in prolactin (Rosenfield & Ehrmann, 2016). A lot of studies have exposed that ROS level is significantly increased in patients with PCOS compared to healthy women (Lu *et al*., 2007; Murri *et al*., 2013).

Some studies evaluated gene expression of Cu/Zn SOD and MnSOD in PCOS women in follicular fluid (Seleem *et al.,* 2014) and placental tissue samples and demonstrated increased SODs gene expression. Although both superoxide dismutase enzymes (Cu/zn SOD and Mn SOD) participate in the same metabolic pathway, their locations and transcriptional regulation differ (Zelko *et al*., 2002). *SOD1* expression is

steady, and its products control reactive species turnover in the cytoplasm, allowing cells to maintain homeostasis. SOD2 expression, on the other hand, is sensitive to various internal and external stimuli and serves as the primary defence against oxidative stress within mitochondria.(Williams & Kwon, 2004). Some studies on other women's diseases, such as the (Donabela *et al*., 2015) study, found a significantly higher expression of *SOD1* in women with endometriosis.

2.9. Transcriptional factors involved in the regulation of the *sod* genes

Many transcriptional regulatory elements in the proximal promoter regions of the *sod* genes that are binding sites for several common transcription factors, including NF-κB, AP-1, AP-2, Sp1, and C/EBP, which play essential roles in regulating the constitutive or inductive expression levels of all three SODs**.**

- **NF-κB** acts as a regulator of genes by serving as an immediate response to injurious cellular stimuli. found in both promoter and intronic regions of all three *sod* genes (Oeckinghaus & Ghosh, 2009). Because the *sod1* gene is frequently constitutively expressed and not as easily inducible as other superoxide dismutases, it is considered a (housekeeper gene) and is occasionally used as an internal control to compare variations in MnSOD expression level or activity (Minc E *et al*.,1999)
- **Specificity Protein 1 Sp1** is a zinc-finger protein that acts as a transcription factor by binding directly to DNA through three consecutive zinc-finger domains in the C-terminus and enhances gene transcription (Flashner *et al*., 2022). Multiple GC boxes are the identifiable characteristic of the Sp1-dependent promoter. Thus, the GC-rich motif contained within the three *sod* gene promoters

suggests a common regulatory role of Sp1 in the expression of SODs (Xu Y *et al.*,2002).

- **Activator Protein 1 (AP-1)** AP-1 acts as a transcriptional regulator to modulate signal transduction processes involved in cell proliferation, and transformation (Garces de los Fayos Alonso et al., 2018), the increased DNA binding capacity of AP-1 could cause a reduction in Cu/zn SOD. The activity of AP-1 is topic of redox regulation. Thus, alteration in *sod* genes expression may modulate AP-1 activity (Zhou W *et al*., 2001)
- **Activating Protein 2 (AP-2)** AP-2 is a family of closely related transcription factors consisting of AP-2alpha, AP-2beta, AP-2gamma, AP-2delta and AP-2epsilon (Eckert D *et al.*,2005). In addition to direct binding to the target gene, it can crosstalk with other transcriptional factors to alter the expression of a specific gene (Xu Y *et al.*,2008**).** On the other hand, AP-2 plays a negative role in the constitutively low expression of MnSOD by suppressing Sp1-dependent transcription (Zhu CH *et al.*,2001) **.**
- **CCAAT-Enhancer-Binding Proteins (C/EBP)** C/EBP proteins consist of six members, C/EBP α to C/EBP ξ, which can interact with the CCAAT box motif present in many gene promoters. C/EBP- factors are necessary for *sod1* transcription (Ramji & Foka, 2002)
- **FOXO** The family of forkhead box class O (FoxO) transcription factors includes FoxO1, FoxO3, FoxO4, and FoxO6. FoxOs regulate genes involved in various pathways such as metabolic regulation, cell and tissue homeostasis, and immunity (Calissi et al., 2021).Foxo characterized by a winged helix DNA binding domain known as a Forkhead box.FOXO transcription factors function mostly as

transcriptional activators, and their activity is inhibited by insulin and growth factor signalling (Kaestner *et al*., 2000). During oxidative stress, FoxO4 binds to the promoter of the *SOD2* gene and induces the expression of manganese superoxide dismutase, an antioxidant enzyme located within the mitochondrial matrix (Araujo *et al*., 2011).

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2. Literature Review

2.1. Ovarian function

The ovary is the essential organ of the female reproductive system. It has two main functions: the production of gametes or oocytes and the secretion of signalling and regulatory substances, including those that influence other parts of the reproductive system in controlling female maturation, gamete production, regularity of cycle and behavior, and those, which support embryo development, gestation, and lactation. The ovary has two structural regions: the outer cortex contains follicles at various stages of development together with structures derived from follicles, while the inner medullar consists mainly of stromal tissues and vascular elements (Figure 2.1)(Sirotkin & Luck, 2012)

Several factors can affect ovarian morphology and physiology, like polycystic ovary syndrome, which is further exacerbated by obesity, in which Ovarian volume and follicle number increase in women with PCOS(Alsamarai *et al.*, 2009). Furthermore, the incidence of uterine myomas (fibroids) and endometriosis (a progressive pelvic inflammatory disorder) is the most common gynaecological diseases affecting women's quality of life and ovaries function (Uimari *et al*., 2021). In addition, several genetic factors may limit the “ovaryan reserve” an expression of the total number of oocytes within the ovary; in which many genes are involved in ovary development and function and many more are vital participants in the implicated pathways(Pelosi *et al.*, 2015). Moreover, environmental factors may also influence reproduction and ovary development. There are suggestions that dioxin exposure is linked to endometriosis, phthalate exposure may affect ovarian reserve, and bisphenol A can interfere with oocyte progress and maturation(Hart, 2016).

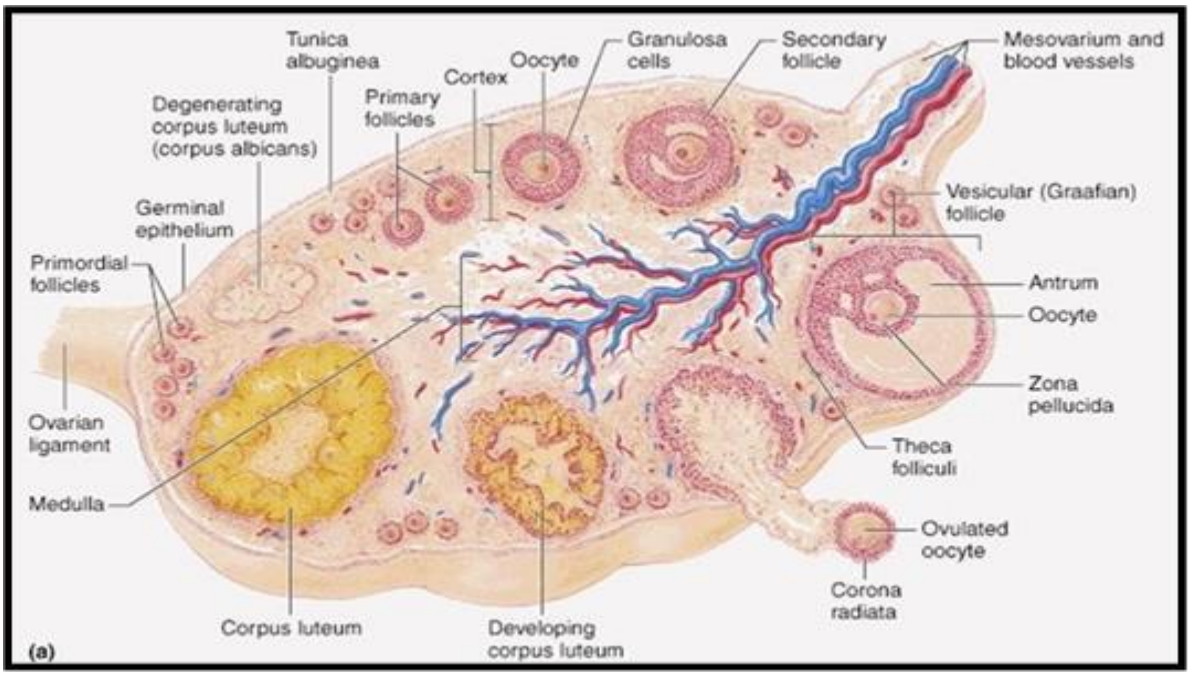


Figure (2.1): The anatomy of the ovary (L Mescher, 2016)

2.2. Polycystic ovarian syndrome (PCOS)

2.2.1. Criteria for diagnosis of PCOS

Polycystic ovarian syndrome is one of the most common endocrine disorders of women of reproductive age and the major cause of ovulatory infertility (Joham *et al*., 2015). It was defined as the change of ovarian morphology by Chereau in 1844 (Chéreau, 1844). The European Society established the diagnostic criteria for Human Reproduction and Embryology (ESHRE) and the Americans Society of Reproductive Medicine (ASRM) in 2003 based on extensive studies during the last decades, which is then so-called Rotterdam Consensus Criteria(Fauser *et al*., 2012). It was first described by Stein and Leventhal as a syndrome of oligomenorrhea and polycystic ovaries, which was variably attended by hirsutism, acne, and obesity (Stein, 1935). Later, biochemical and radiographic imaging was assumed to assist in diagnosis (Bachanek *et al*., 2015).

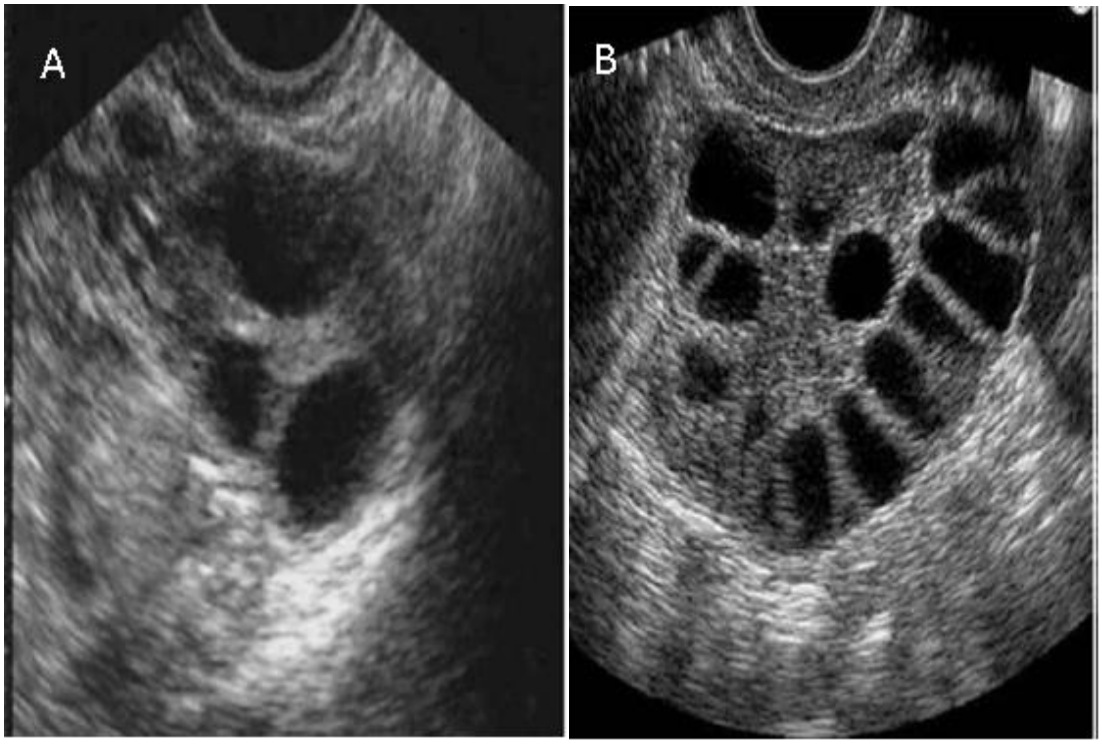
In early 1970, the scientific community studied increased serum levels of LH elevated LH/FSH ratio, and changed function in the hypothalamic–pituitary–ovarian axis that can be associated with PCOS pathology (Rebar *et al*., 1976) Moreover, The ultra-sonographic finding of polycystic ovaries was first used in 1981 as one diagnostic criterion of PCOS in the visualizing and diagnosis of polycystic ovaries (Swanson *et al*., 1981).

Then in 2003, the Rotterdam Criteria used ultrasound as a new criterion to be added to the two previous criteria. The Europeans Society of Human Reproductions and Embryology/American Society for Reproductive Medicine Rotterdam consensus developed the diagnosis of PCOS, requiring two of three features: anovulation or oligo ovulation, clinical and/or biochemical hyper androgens and polycystic ovarian morphology seen on ultrasound. Finally, In 2006, the androgen Excess and PCOS Society (AE-PCOS) underlined PCOS to be regarded as a condition of androgen excess and defines the syndrome as hyperandrogenism together with oligo/anovulation and polycystic ovaries, which is the third criterion (Azziz *et al.*, 2006).

Exclusion of other androgen excess conditions should be excluded such as non-classical congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumors, hyperprolactinemia, thyroid diseases, drug-induced androgen and other causes of oligomenorrhea or anovulation (Spritzer, 2014). Also, the National Institutes of Health Criteria (NIH) have been proposed some criteria for the diagnosis of PCOS in 1990 that include the only presence of clinical and biochemical hyperandrogenism and oligomenorrhea anovulation (Zawadeski & Dunaif, 1992)**.**

The syndrome acquired its name due to multiple ovarian cysts diagnosed using ultrasound by the presence of eight or more follicular cysts, usually with an average size of 2-9 mm in diameter arranged around a dense stroma within the PCOS ovary. The combination of multiple follicles and an

increased amount of stroma contribute to the overall increase in the ovarian size (ovarian volume >10cm^3), which is 1.5 to 3 times larger than normal (Balen, 2004) (Figure 2.2)



Normal *ova*ry **Polycystic ovary**

Figure (2.2): Comparison of normal and polycystic ovary (Hiremath & Tegnoor, 2013).

2.2.2. Clinical features of PCOS

Polycystic ovary syndrome (PCOS) is an endocrine disorder that has multiple potential etiologies and variable clinical symptoms in women of reproductive age (Ercan *et al.*, 2013).clinical and Biochemical hyperandrogenism of ovarian origin and to a lesser extent adrenal is evident for about 60–80% of PCOS patients, resulting in one of the main features of this syndrome (Franks, 2006). Ovarian hyperandrogenism is due to a defect in the intrinsic steroid synthesis in ovarian thecal cells which results in high levels of LH and low levels of FSH which consider the most stable and obvious diagnostic feature of PCOS that is evaluated clinically by

hirsutism (Hirsutism , excessive growth of terminal hair on the face and body of a female in a typical male pattern distribution ,acne and alopecia(Diamanti-Kandarakis *et al.*, 1999; Somani *et al.*, 2008). A recent report by the Androgen Excess PCOS Society analyzed 18 studies from 1983 to 2007, including 6281 women with PCOS, and found that 74.7% of women have hirsutism(Azziz *et al.*, 2009). Hirsutism in these women is generally more severe in the context of obesity, particularly the abdominal phenotype (Carmina, 2006; Gambineri et al., 2002; Wild, 2004).

Menstrual abnormality is the most important feature of the polycystic ovarian syndrome, which includes oligomenorrhea (infrequent menses with less than nine menstrual periods per year), amenorrhea (absence of menstruation for > 6 months without being pregnant), and irregular bleeding (loss of the cyclic menstrual pattern) (Hoeger *et al*., 2014).

PCOS associated with an increased risk of metabolic disorders that include insulin resistance(IR) and hyperinsulinism, diabetes mellitus, cardiovascular disease ,dyslipidemia, and endometrial carcinoma(Azziz et al., 2004). Another common feature of PCOS is obesity, Approximately 50% of PCOS women are overweight or obese, which plays a pathogenetic role in the development of the syndrome in susceptible individuals, obese PCOS women have elevated hyperandrogenism and related clinical features like hirsutism, anovulation and menstrual abnormalities than normal-weight PCOS women. This picture tends to be more distinct in obese PCOS women with the abdominal phenotype (Gambineri *et al.*, 2002).Along with impaired metabolic and reproductive features, Stress and depression are considered high-risk factors. This high level of stress and anxiety in patients with PCOS may be due to various reasons such as obesity, hirsutism, alopecia, and infertility (Sadeeqa *et al.*, 2018)

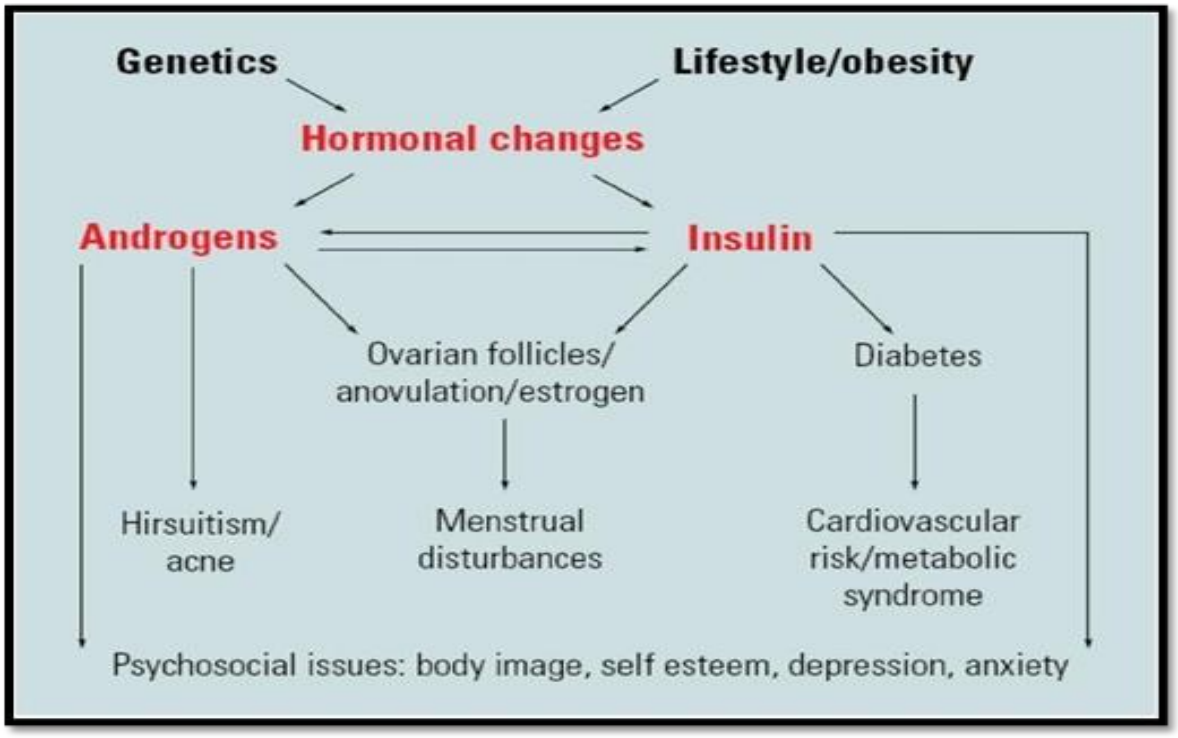


Figure (2.3): Scheme of aetiology and clinical features, including menstrual, cardiovascular/metabolic and psychosocial issues of a polycystic ovarian syndrome (Teede *et al*., 2010)

2.2.3. Prevalence of PCOS globally and locally

Polycystic ovary syndrome (PCOS) is a multifactorial disorder that has significant metabolic, reproductive and psychological consequences (Teede *et al*., 2010)It is a major public health concern affecting 6-10% of reproductive-aged women worldwide (Bozdag *et al*., 2016)The intricacy and difficulties of the existing diagnostic criteria must be recognized to determine the prevalence of PCOS in subgroup populations. There are three sets of diagnostic criteria used: those established by the National Institutes of Health (NIH) international conference on PCOS in 1990, the European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine (ESHRE/ASRM) in 2003 (referred to as the Rotterdam criteria), and the Androgen Excess Society and PCOS Society (AE-PCOS) in 2006 (Garad *et al*., 2011). To assess the presence or absence of PCOS, each set of criteria has slightly distinct

clinical, biochemical, and image-based results (Okoroh *et al.*, 2012). Changes in diagnostic criteria have a significant impact on the prevalence of PCOS. Prevalence rates have been recorded as low as 1.6 percent using all three criteria (Okoroh *et al.*, 2012) and as high as 18 percent using the Rotterdam criterion (Lim *et al.*, 2013) in similar populations. According to Futterweit's statistics, 50-75 percent of women with PCOS in the world are ignorant that they have the condition which may affect the prevalence statically analysis (Futterweit, 1999). Furthermore, various factors influence PCOS prevalence, including age, hormonal profile, lifestyle, smoking, physical activity, race, and geographical distribution, which vary from one population to another (Motlagh Asghari *et al.*, 2022)

In 2017, 1.55 million (95%) incident cases of PCOS among women of reproductive age (15–49 years) were reported globally, representing an increase of 4.47% (2.86–6.37%) from 2007 to 2017. The global age-standardized incidence rate of PCOS among women of reproductive age was 82.44 (64.65–100.24) per 100 000 population in 2017, which represents an increase of 1.45% (1.43–1.47%) from 2007 to 2017 (Liu *et al.*, 2021).

In Iraq, there are no accurate studies on the prevalence of PCOS, only one local study that was carried out in the north of Iraq, found that the prevalence of PCOS was 6.11% in the gynaecology out-patient visits and 35.39% among infertile women observed among infertile women attending In vitro fertilization (IVF) centre using the Rotterdam 2003 criteria for diagnosis (Hussein & Alalaf, 2013).

2.2.4. Pathogenesis of PCOS

polycystic ovary syndrome (PCOS) is considered a heterogeneous collection of signs and symptoms that form a range of a disorder with a mild presentation in some but a severe disturbance of reproductive, endocrine and metabolic function in others. PCOS pathophysiology appears to be multifactorial and polygenic, and many of theories were proposed to explain the pathogenesis of PCOS (Soni *et al.*, 2018):

First; A change in gonadotropin-releasing hormone secretion results in increased luteinizing hormone (LH) secretion is characteristic of the hallmark of PCOS. LH is secreted in a pulsing routine. PCOS women have an increase in the LH pulse frequency and capacity, resulting in increased 24-hour secretion. This increase in LH secretion is thought to occur as a result of the increased frequency of hypothalamic gonadotropin-releasing hormone (GnRH) pulses which leads to an increase in androgen production by the theca cells within the ovary(Patel *et al.*, 2003)

Second; A defect in androgen synthesis due to an increase in ovarian androgen production**.** The increase in LH with hyperinsulinemia leads to an increase in androgen production by ovarian theca cells (Tsilchorozidou *et al., 2004)*. Moreover, the most likely primary factor leading to an increase in testosterone secretion in PCOS is an increase in ovarian enzymatic activity involved in the synthesis of testosterone precursors (Hill, 2003)

Third; An alteration in insulin secretion and insulin action results in hyperinsulinemia and insulin resistance (IR) which is defined as (reduced glucose response to a given amount of insulin), and appears to be the common pathway of disease amongst women with PCOS. Increasing in the circulating levels of insulin has a direct effect on the ovaries by releasing other factors such as growth factor 1 (IGF-1) from the liver which employs an effect on the ovary. Moreover higher levels of insulin and IGF-1 in the

ovary leading to the release of higher levels of testosterone. All of these hormones (including insulin, IGF-1 and testosterone) prevent the growth of ovarian follicles during the ovulation, leading to an accumulation of small ovarian follicles less than 10 mm diameter that do not progress through the ovulation(Johnson, 2014)

In PCOS, Hyperinsulinemia, hyperandrogenemia and altered intraovarian paracrine signaling can disrupt ovarian follicle growth, The consequent follicular arrest in PCOS is accompanied by anovulatory, menstrual irregularity ,subfertility and the accumulation of small antral follicles within the ovary, giving it a polycystic morphology (Goodarzi *et al.*, 2011) (Figure 2.4).

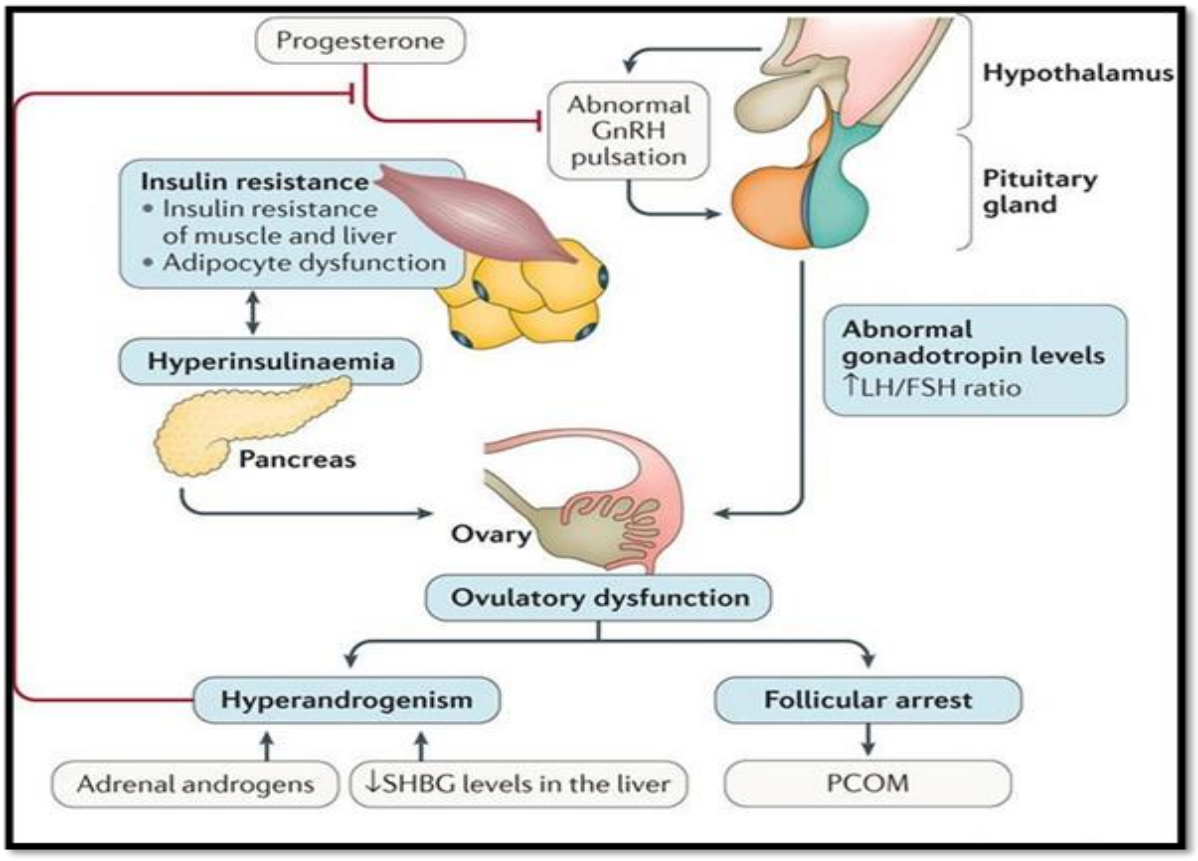


Figure (2.4): The pathophysiology of polycystic ovary syndrome (Taffy *et al*.,2019)

2.3. Hormones associated with PCOS

2.3.1. Luteinizing Hormone (LH)

The pituitary gland's anterior lobe is stimulated by (GnRH) to produce LH. At midcycle, there is an LH surge that induces an increase in maturation-promoting factor concentrations, causing oocytes to complete meiosis I and begin meiosis II; it also increases progesterone production by follicular stromal cells (luteinization); and it causes follicular rupture and ovulation (Nedresky & Singh, 2021).

In conjunction with Follicle Stimulating Hormone (FSH), luteinizing hormone promotes follicular growth and ovulation. As a result of the complimentary activity of FSH and LH, proper follicular expansion occurs (Raju *et al.*, 2013). In addition, LH operates in the ovary after ovulation to maintain the corpus luteum's release of predominantly progesterone to prepare the uterus for future implantation (Palomba *et al.*, 2015).

In PCOS women, abnormality of the hypothalamic-pituitary-ovarian axis has been compulsory in the pathophysiology of polycystic ovarian disease. disorder in the secretion pattern of the gonadotrophin-releasing hormone (GnRH) results in a relative increase in LH to FSH release (Saadia, 2020). Usually, in healthy women, the ratio between LH and FSH lies between 1 and 2. In polycystic ovary disease women, this ratio becomes reversed, and it might reach as high as 2 or 3; as a result of raised LH/FSH ratio, ovulation does not occur in polycystic ovary disease patients (Johansson & Stener-Victorin, 2013). Many studies have been reported on LH elevation in PCOS patients as (Deswal *et al*., 2019)and found a significant change in the level of LH hormone in PCOS patients. Moreover, oxidative stress in PCOS patients can play a vital role in LH,

FSH secretion and hence the evolution process in which oxidative stress causes anxiety, depression and mitochondrial dysfunction. The Hypothalamus pituitary axis is stimulated by stress, triggering the excess secretion of cortisol from the adrenals, releasing the inflammatory markers with increased lipid peroxidation and oxidative stress (OS).cortisol secretion suppresses the gonadotropin-releasing hormone (GnRH), which then decreases the release of luteinizing hormone (LH) and follicular stimulating hormone (FSH). Failure to stimulate ovaries to secrete estradiol and with mitochondrial dysfunction, oocyte maturation failure occurs in PCOS (Alam *et al.*, 2019).(Figure 2.5)

Figure(2.5): Effect of oxidative stress on LH,FSH secretion, and oocyte maturation (Alam *et al.*, 2019)

2.3.2. Follicle-Stimulating Hormone (FSH)

The hypothalamus produces gonadotropin-releasing hormone (GnRH), which acts on pituitary gland cells in the anterior lobe (adenohypophysis) to secrete gonadotropins. These hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), stimulate and regulate ovarian cyclic changes. FSH stimulates the growth of approximately 15 to 20 primary-stage (preantral) follicles at the start of each ovarian cycle. Although the presence of FSH is not required to promote the development of primordial follicles to the primary follicle stage, these primary follicles die and become atretic if it is not present (Marques *et al.*, 2022).

LH and FSH are the hormones that encourage ovulation and are secreted by the pituitary gland in the brain. Most women have equal amounts of LH. and FSH during the early part of their cycle. However, there is an LH. Surge in which the LH amount increases 24 hours before ovulation occurs. Once the ovary releases the egg, the LH levels go back down. While many women with PCOS have LH level is often two or three times that of the FSH level. This situation is called an elevated LH to FSH ratio or a ratio of 3:1. This change in the LH to FSH ratio is enough to disrupt ovulation. While this used to be considered an essential aspect in diagnosing PCOS (Raju *et al*., 2013)

Regulation of oxidative stress (OS) is important to prevent damage to female reproductive physiology. While normal OS levels may have a regulatory role, high OS levels may negatively affect vital processes such as folliculogenesis.(Martín-Ramírez *et al.*, 2021). FSH is a glycoprotein released by the pituitary gland that plays an important role in encouraging follicle development and regulating ovarian function during folliculogenesis (Huang *et al.*, 2019). FSH is a key survival factor for antral follicles, and it has been suggested that it can boost granulosa cell (GC)

tolerance to oxidative stress during follicular apoptosis(Shen *et al.*, 2014). FSH has been proposed to play a unique role in protecting the ovary from oxidative damage (Shen *et al.*, 2017). fertility diseases like endometriosis and polycystic ovary syndrome, contribute to increased OS levels and enhance the impact of OS on female reproduction (A. Agarwal *et al.*, 2012). Previous studies in women who have fertility treatments showed a relationship between higher oxidative stress biomarker levels and lower oocyte fertilization potential (Tamur*a et al.*, 2008).

2.4. Treatment of PCOS

Management of PCOS was based on the type and extent of the disorders and if there is a need for pregnancy, the goals of the therapeutic program include improvement in menstrual cycles, reduce circulating androgens, reduce insulin resistance, prevent metabolic complications, decrease cardiovascular risk, weight loss achievement, improving the response to ovulation induction therapy, treatment of infertility and prevent endometrial carcinoma (Legro, 2015)

The First line in the treatment of PCOS is the non-medication approach, which includes lifestyle modification through diet and exercise. There is a significant correlation between hyperinsulinemia and obesity in PCOS, which is mostly associated with a decrease in ovarian function. Weight loss may help to improve IR by decreasing its harmful effect on the hormonal disorder, ovulation and menstrual regularity (Saleem & Rizvi, 2017)

Medical treatment of PCOS can be divided into two components depending on the type of case, one of which is considered as acute case and need control of irregular menses and treatment of hirsutism, other is more chronic which needs the management of infertility (Artini *et al.*, 2010). Irregular menses can be controlled by oral contraceptives, which are effective in normalizing menstrual cycles and decreasing bioavailable

testosterone levels to minimize hirsutism (Diamanti-Kandarakis et al., 2003).

Typically, treatment of PCOS focuses on insulin-sensitizing agents which including metformin and thiazolidinediones such as troglitazone, which aid the reduction of insulin resistance and subsequent hyperinsulinaemia which is used to assist insulin sensitivity and reduction of androgen levels(Arlt *et al.*, 2001). This has also been seen with pioglitazone and rosiglitazone but is only effective at supra-physiological concentrations. The androgen-lowering potential of those TZDs in current clinical use may therefore be effective through a more indirect mechanism (Balen, 1993).

2.4.1. Metformin

Metformin or Glucophage (commercial name) has a chemical structure ($C_4H_{11}N_5$) as shown in figure (2.6). It is the first-line drug of choice for the treatment of Type 2 diabetes mellitus (T2DM), particularly in overweight and obese people(Nasri & Rafieian-Kopaei, 2014). Metformin acts by refining the sensitivity of peripheral tissues to insulin which results in a reduction of circulating insulin levels and increases glucose uptake by peripheral tissues leading to a decrease in insulin resistance (IR)(Bailey, 1996; Dmitri *et al.*, 2002).

Metformin has been seen to reduce hyperandrogenemia and the direct mechanisms behind this, it is indirectly linked to both the reductions in circulatory insulin and the possible inhibition of steroidogenic enzymes. Research has shown that metformin stimulates adenosine monophosphate-activated protein kinase (AMP-K) a major cellular regulator of lipid and glucose metabolism. In doing so glucose uptake is increased (Zhou *et al.*, 2001). Additionally, metformin has been seen to alleviate glucose toxicity aiding glycemic control and lipid regulation (DeFronzo *et al.*, 1995). In

some cases, metformin can inhibit the effects of CYPl7 and may therefore explain reductions in androgen levels (La Marca *et al*., 2004) however contradictory research in human studies has shown that no effect is seen suggesting any reductions in androgen is secondary to the effects metformin has on insulin levels (Arlt *et al.*, 2001).

Side effects of metformin included gastrointestinal symptoms such as nausea, diarrhoea, flatulence, bloating, anorexia, metallic taste, and stomach pain. These symptoms can be decreased in patients to varying degrees by gradually administering metformin and titrating the dose increase based on the severity of symptoms(Nestler, 2008). Velazquez and colleagues reported in an observational study a significant improvement in menstrual regularity and reduction in circulating androgen levels (Velazquez *et al.*, 1994)as well as a significant reduction in body weight, which confounded their findings.

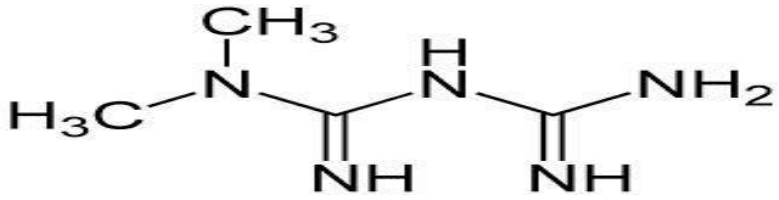


Figure (2.6): Metformin structure (Rizvi *et al.*, 2015)

2.5. Oxidative stress

Oxidative stress (OS) can be defined as an imbalance between increased levels of reactive oxygen species (ROS) and a low activity of antioxidant mechanisms. They are normally generated as by-products of oxygen metabolism, mainly by mitochondria, during physiological and pathological conditions in which $O_2^{\bullet -}$ can be formed by cellular respiration. Also, they are required for cell function, including the production of energy by the mitochondria. An increase in oxidative stress can induce damage to the cellular structure and possibly destroy tissues (Preiser, 2012).

ROS are the active form of oxygen that is needed for several cellular functions and in response to different stimulation, the diatomic oxygen molecule (O2) does not react spontaneously with other molecules as it contains two unpaired electrons. The reaction of oxygen with organic molecules was achieved either by oxidation or reduction(Magder, 2006), and affected several of the cellular compounds, which can lead to:

1. DNA modifications by degradation of bases, single or double-stranded DNA breaks pyrimidine, purine, or sugar-bound modifications, mutations, deletions, translocations and cross-linking with proteins which highly related to carcinogenesis, ageing, and neurodegenerative, cardiovascular, and autoimmune diseases. Also affects Promoter regions by attacking the transcription factor–binding sites and can modify the binding of transcription factors and thus change the expression of related genes (Ghosh & Mitchell, 1999)

2. lipid peroxidation disturbs the membrane lipid bilayer arrangement which leads to the inactivation of membrane-bound receptors and enzymes as well as increased tissue permeability(Girotti, 1998). Products of lipid peroxidation, such as MDA and unsaturated aldehydes, can inactivate several cellular proteins by forming protein cross-linkages that cause depletion of intracellular GSH and induces peroxide production (Keller *et al.*, 1997).

3. Protein denaturation by fragmentation of the peptide chain, alteration in electrical charge of proteins, and oxidation of specific amino acids that lead to increased exposure to proteolysis and degradation(Kelly & Mudway, 2003), Enzymes that have metals on their active sites are mainly more sensitive to metal oxidation and modification of enzymes which leads to inhibit their activities(Stadtman, 1990). protein modification has been

directly associated with the development of human diseases, such as Alzheimer's, autoimmune diseases, Crohn’s disease, kidney diseases, and Parkinson’s disease (López-Alarcón *et al.*, 2014)

Oxidative stress includes exogenous factors, such as cigarette smoke, ozone exposure, ion radiation, alcohol consumption, strenuous physical activity, poor diet, and Endogenous factors like the oxidative burst from activated macrophages, producers from ROS NADPH oxidase in cell membranes, mitochondria, peroxisomes (Birben *et al.*, 2012).

The imbalance between pro-oxidants and antioxidants and oxidative stress can lead to several reproductive diseases such as polycystic ovary syndrome (PCOS), endometriosis, and unexplained infertility. Endometriosis, polycystic ovarian syndrome (PCOS), and unexplained infertility can be caused by an imbalance of pro-oxidants and antioxidants, as well as oxidative stress(Tesarik, 2021). In reaction to oxidative stress, pregnancy problems such as spontaneous abortion, recurrent pregnancy loss, and hypertension can develop. Excessive body weight and lifestyle factors such as cigarette smoking, alcohol use, and recreational drug use have been found to induce excess free radical generation, which may impact fertility. Environmental toxins are causing oxidative states, which may contribute to female infertility (Ashok Agarwal *et al.*, 2012).

Oxidative stress has been associated with PCOS. According to meta-analysis research, circulating markers of oxidative stress are abnormal in women with PCOS, suggesting that oxidative stress plays a role in the pathogenesis of PCOS. Oxidative stress has a role in PCOS by altering steroidogenesis in the ovaries, which leads to increased androgen levels, disrupted follicular development, and infertility (Sulaiman *et al.*, 2018). Furthermore, obesity, insulin resistance, and cardiovascular risks have been linked to oxidative stress in PCOS women (Lorenz & Wild, 2007)

2.5.1. Free radical

Free radicals are the products of normal cellular metabolism, which can be defined as an atom or molecule, that have one or more unpaired electrons in the outer orbit. The odd number of electrons of a free radical makes it unstable, short-lived and highly reactive, therefore, it can catch electrons from other compounds to reach stability. As a result, these molecules lose their electrons and become free radicals, triggering a chain reaction cascade that eventually damages the living cell(Phaniendra *et al*., 2015). ROS and RNS play a vital role as useful and toxic compounds to the living system. At the moderate level, they are involved in various physiological functions such as immune function, cellular signalling pathways, mitogenic response and in redox regulation (Valko *et al.,* 2007), While at higher concentrations generate oxidative stress and cause potential damage to the biomolecules, as oxidative stress is developed when there is an excess production of ROS and RNS or in deficiency of enzymatic and non-enzymatic antioxidants (Phaniendra *et al*., 2015).

ROS and RNS collectively constitute both the free radicals and non-radical reactive species:

The radicals include Superoxide ($O^{\cdot-}_2$), Oxygen radical ($O^{\cdot\cdot}_2$), Hydroxyl ($OH^{\cdot}$), Alkoxyradical ($RO^{\cdot}$), Peroxyl radical ($ROO^{\cdot}$), Nitric oxide ($NO^{\cdot}$) and nitrogen dioxide ($NO^{\cdot}_2$)(Hilali *et al*., 2013).

The non-radical species include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), singlet oxygen ($^1O_2$), nitrous acid (HNO_2), nitrosyl cation (NO^+), nitroxyl anion (NO^-), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), nitronium cation (NO_2^+), organic peroxides (ROOH), aldehydes (HCOR) and peroxynitrite (ONOOH)(Kohen & Nyska, 2002). These non-radical species are not free radicals but can simply lead to free radical reactions in living organisms(Genestra, 2007).

2.5.2. ROS in normal physiology

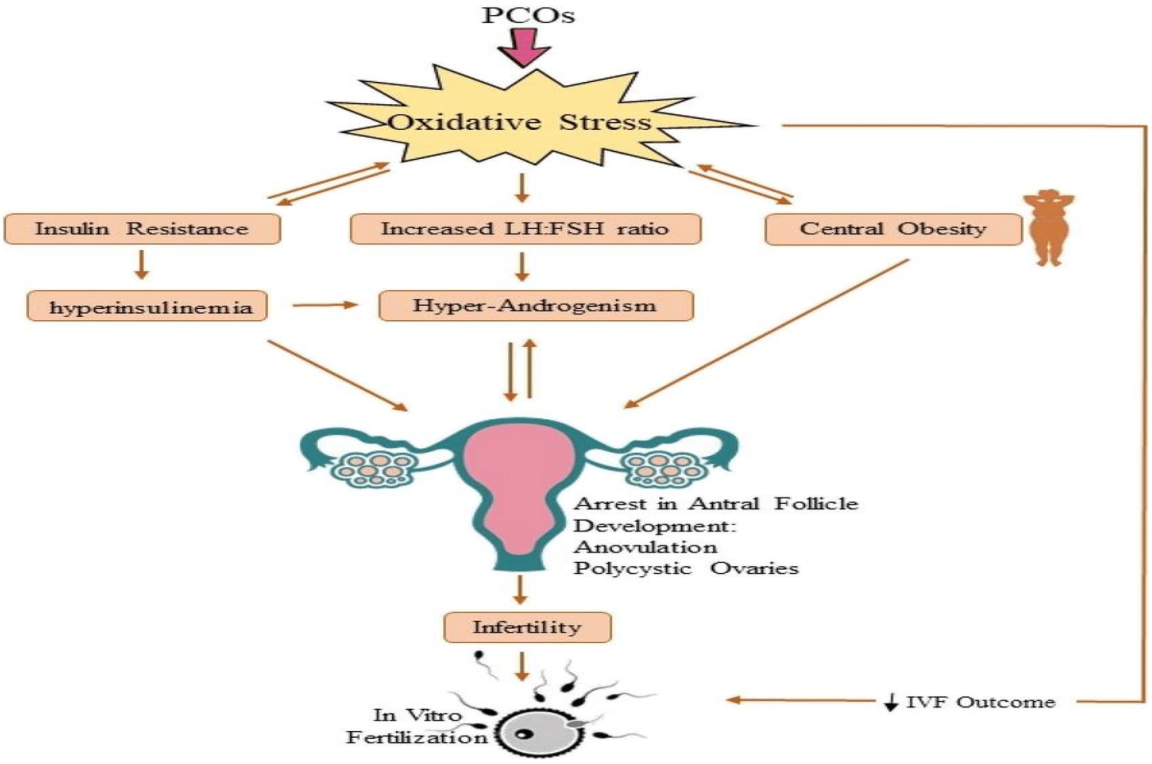
Normally, a low concentration of ROS is necessary for normal physiological functions such as gene expression ،cellular growth, and defence against infection.ROS serve as secondary messengers in many developmental stages of prenatal and embryonic growth in mammals (Kunwar & Priyadarsini, 2011). ROS participate in the biosynthesis of molecules such as prostaglandin that accelerate developmental processes and thyroxin synthesis that, regulated by H_2O_2 concentration, catalyze the binding of iodine atoms to thyroglobulin (Shulaev & Oliver, 2006). Finally, ROS are involved in the immune system via inducing the proliferation of T cells, which were admitted to generating ROS to destroy bacterial cells for engulfing by the phagocyte.

2.6. Oxidative stress and PCOS

Polycystic ovarian syndrome (PCOS) is a highly complex and heterogeneous endocrine metabolic disorder, characterized by oligo-ovulation or anovulation, biochemical or clinical hyperandrogenism, presence of polycystic ovaries and associated with metabolic disorders, including insulin resistance, obesity and diabetes (Rotterdam.2004). Several studies have found that when the oxidative state is evaluated using circulating markers such as superoxide dismutase (SOD) and catalase, the OS level in PCOS patients is much higher than in the normal community. However, obesity, insulin resistance, hyperandrogenemia, and chronic inflammation are all found to be strongly linked with OS level (Murri *et al*., 2013)

The profound factors in PCOS that increase oxidative stress and obesity, IR, and hyperglycemia; however, non-obese PCOS women without IR are also reported to increasing oxidant status (Verit & Erel, 2008), suggesting that other factors may also contribute to inducing the

production of ROS in these women, Recent studies found that the elevation of circulating androgens is associated with high oxidative stress in women with or without PCOS (Gonzalez *et al.*, 2012). Oxidative stress appears to be involved in PCOS by causing altered steroidogenesis in the ovaries, which subsequently contributes to increased androgen levels, disturbing follicular development, and infertility (Sulaiman *et al.*, 2018). Elevated levels of ROS and reduced antioxidant capacity are closely related to reduced oocyte maturation and low embryo quality. As these molecules may reduce oocyte quality by changing the equilibrium of follicular fluid in the follicular microenvironment (Gharaei et al., 2021). (figure 2.7)



Figure(2.7):Relationship between oxidative stress and PCOS complications (Gharaei *et al.*, 2021).

2.7. Antioxidant system

Antioxidants are structurally diverse groups of small organic molecules and enzymes that form complex systems of overlapping activities. Act synergistically to improve cellular defence and counter oxidative stress

caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). Antioxidants are classified into enzymatic and non-enzymatic antioxidants. The non-enzymatic antioxidants intercept and terminate free radical chain reactions. Such as vitamins E, A, and C, flavonoids, carotenoids, glutathione, plant polyphenols, uric acid, curcumin, melatonin, and bilirubin(Agarwal *et al.,* 2008). Vitamin C and Vitamin E were included in this study:

1. Vitamin C or ascorbic acid is the major non-enzymatic water-soluble antioxidant in the biological system, which acts as a cofactor in several metabolic reactions required for a wide range of biological functions. Humans lost the capability to synthesize ascorbic acid due to a defect in L-gulono-1,4-lactone oxidase that catalyzes the transformation of L-glucono lactone into ascorbic acid, therefore humans depend on the diet as a source for vitamin C to prevent the vitamin C deficiency. Vitamin C can be attributed to several biological functions as a cofactor for a numeral of enzymes like hydroxylases that are involved in collagen synthesis and as a water-soluble antioxidant (Traber & Stevens, 2011)

Vitamin C generally works as an antioxidant by directly reacting with ROS and has a vital role in defences against oxidative stress and preventing oxidative damage to important biological macromolecules such as DNA, lipids, and proteins and reduces redox-active transition metal ions in the active site of specific biosynthetic enzymes(Carr & Frei, 1999). The oxidized vitamin C, dehydroascorbic acid (DHA), stimulates the antioxidant defences of cells(Puskas *et al*., 2000).

Vitamin C plays a vital role in regulating the menstrual cycle and ovarian function in which ascorbic acid excretion declines directly before ovulation, then increases again after the temperature rises post-ovulation. Ascorbic acid levels are stimulatory to progesterone hormones and have

been found in high concentrations in the corpus luteum (Varshney & Kale, 1990). Vitamin C in the ovaries is responsible for collagen synthesis, which is required for follicle and corpus luteum growth. Problems in this function may contribute to the progress of ovarian cysts and PCOS(Chen *et al.*, 2005)

2. Vitamin E, a lipid-soluble substance known as tocopherol, was found by Evans and Bishop in 1922 (Evans & Bishop, 1922). Vitamin E can antagonize the oxidative stress caused by the oxygen free radicals and antioxidant imbalance by rapidly transferring its phenolic hydrogen atom to neutralize free radicals and regulate the normal physiological function of the reproductive system. vitamin E can reduce the oxidative stress reaction that may have a harmful effect on the number and quality of oocytes(Tarín *et al*., 2002). A lack of vitamin E can cause female infertility, eclampsia, miscarriage, premature delivery, fetal intrauterine growth restriction, and other pregnancy-related diseases(Hubalek *et al.*, 2014)

While enzymatic antioxidants function by converting oxidized metabolic products in a multi-step process to hydrogen peroxide (H_2O_2) and then to water using cofactors such as iron, zinc, copper, and manganese superoxide dismutase(SOD), catalase, and GPx are examples of enzymatic antioxidants. (Agarwal *et al.,* 2008)Antioxidants prevent and limit the adverse effects of oxygen radicals and have essential roles in the female reproductive system as well as in the pathogenesis of female infertility (Kusçu NK and Var A .2009). Changes in the antioxidant concentrations in serum and peritoneal fluid have been studied in idiopathic infertility, tubal infertility, and endometriosis patients. Investigations of antioxidant concentrations in PCOS patients are promising and numerous studies have measured antioxidant markers to correlate ROS and PCOS and the various clinical indicators of metabolic syndrome, including diabetes, obesity, and cardiovascular diseases (Jozwik *et al.,* 1999).

Oxygen species are critical participants in several diseases such as virus infections which cause airway epithelial inflammation, progression to cancer, neurodegenerative processes that include cell death, motor neuron diseases and axonal injury, and both infarction and brain oedema (Uttara *et al.*, 2009). Tissues were protected from this oxidative injury by expressing stress-response genes and genes encoding antioxidant enzymes (MatÉs *et al.*, 1999).

2.7.1. Superoxide dismutase enzyme (SOD enzyme)

In 1967 biochemists, Irwin Fridovitch of Duke University and Joe McCord discovered the antioxidant enzyme SOD, which provides an essential cellular defence against free radical damage. This discovery prompted medical scientists to begin to look seriously at free radicals (Pillai & Pillai, 2002). Antioxidant enzymes include superoxide dismutase, catalase, glutathione peroxidase, and recently appreciated thioredoxin. All of the enzymes play vital roles in the modulation of oxidative stress; the primary role in the superoxide anion radical metabolism is employed by superoxide dismutase, which catalyzes the dismutation of superoxide to hydrogen peroxide (Faraci & Didion, 2004).

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

There are three major forms of superoxide dismutases, MnSOD enzyme that is present in mitochondria, Cu/znSOD enzyme in the cytoplasm, and extracellular SOD (EC-SOD) enzyme, which is secreted by vascular cells into the extracellular matrix.

Superoxide dismutase SOD (EC 1.15.1.1) is an enzyme that catalyzes the dismutation of the superoxide (O^-_2) radical into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Superoxide radical is created as a by-product of oxygen metabolism and, if not regulated, can cause many types

of cell damage (Hayyan *et al.*, 2016); Hydrogen peroxide is then degraded by other enzymes such as catalase. Thus, SOD is an essential antioxidant defence in almost all living cells exposed to oxygen.

2.7.1.1. (Cu\Zn) SOD enzyme

Cu/Zn SOD is a metalloprotein with a molecular mass of approximately 32 000 kDa and has two protein subunits, each containing a catalytically active copper and zinc atom; each subunit is composed of eight antiparallel β strands that form a flattened cylinder, plus three external loops, with the active site held between the barrel and surface loops(figure2.8). The two subunits are tightly joined back-to-back by hydrophobic and electrostatic interactions. The active sites Cu (II) and Zn(II) lie 6.3 Å apart at the bottom of this long channel; the Zn is hidden, while the Cu is accessible for solvent. The side chain of His61 forms a bridge between Cu and Zn and is coplanar with them (Tainer et al., 1982).SOD1 is found in mammalian cells and is expressed at relatively high levels in blood vessels; the activity of SOD1 accounts for 50–80% of total SOD activity (Horiuchi *et al.*, 2004). In physiological conditions, the superoxide dismutase and the non-enzymatic ROS scavengers such as vitamins E, A, and C maintain a steady state between oxidant and antioxidant systems (Russo *et al.,* 2011).This enzyme requires both Cu and Zn to function biologically, and the lack of Cu causes complete inactivation, which frequently results in the development of diseases in humans(BROWN & Besinger, 1998) . Cu/Zn SOD has significant therapeutic potential and physiological importance. This enzyme's function has been studied in relation to a number of distinct red blood cell (RBC) diseases, such as cystic fibrosis, iron deficiency anemia, oxidative hemolytic anemia, thalassemia, sickle cell anemia, and muscular dystrophy(Mavelli *et al.,* 1984). This enzyme has also been linked in recent research to amyotrophic lateral sclerosis, malign breast

disease, steroid-sensitive nephrotic syndrome, and dengue fever(Stieber *et al.*, 2000). SOD activity is markedly elevated in rheumatoid arthritis, ischemia damage, and cancer, pointing to superoxide-related pathophysiology and the involvement of SOD (Noor *et al.*, 2002).

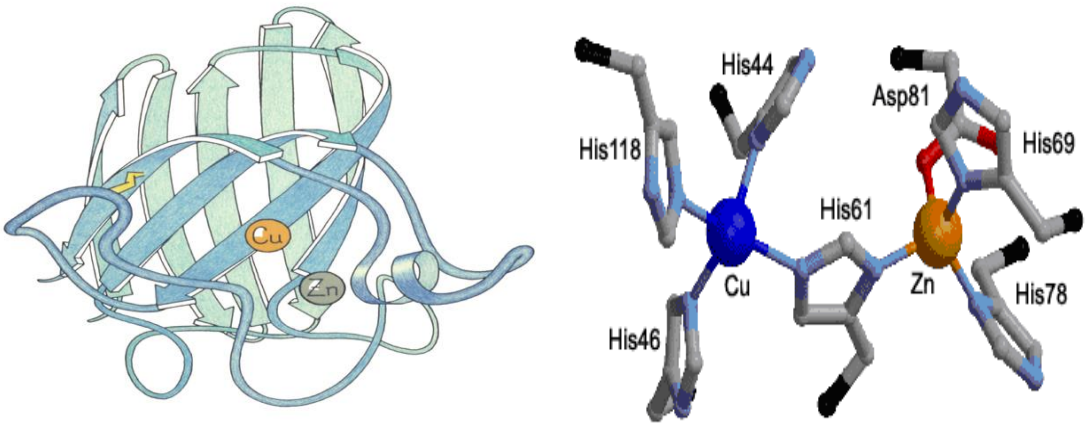


Figure (2.8): The structure of Cu/Zn SOD and the active site (Josko, Osredkar ;and Natasa, Sustar,2011) (Tainer JA *et al.*,1982)

2.7.1.1.1 Genetic structures and organization of the *SOD1* gene

The human *sod1* gene is localized on chromosome 21q22; the latest information on *sod* genes sequences is generated from initial sequencing data and comparative analysis of whole genomic studies among different species, which revealed that the *sod1* gene consists of five exons interrupted by four introns, the variation of intron size found in different studies may be associated with gene polymorphisms in different human tissues and cell lines. The *sod1* promoter has a high GC-rich region, as well as the TATA box and CCAAT box (Levanon *et al.*, 1985).

Oxidative stress is one of the most studied cellular conditions due to its relation to several diseases. When the cells are under oxidative conditions, the antioxidant defences are activated. The levels of expression of the genes related to antioxidant protection are regulated by transcriptional and posttranscriptional mechanisms (Lu *et al.*, 2007). Human SOD1 is a

protein for cellular antioxidant defence. Some studies have focused on better understanding the structure, mechanism, and regulation of human SOD1 mRNA levels under normal and oxidative conditions (Milani *et al.*, 2011).

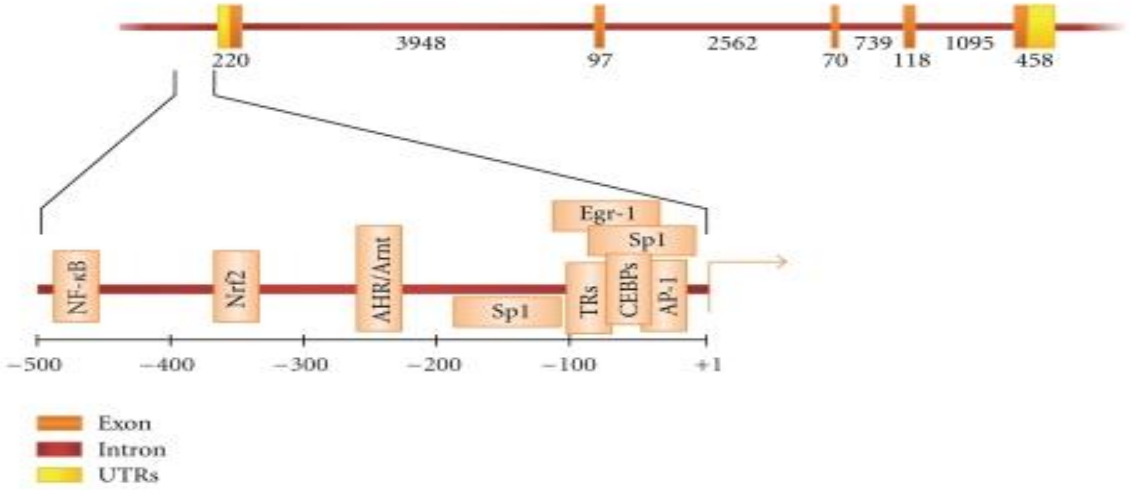


Figure (2.9): Genomic organization of human *SOD1* gene. exons and introns size in base pairs are shown in relationship with each fragment. The 5′ flanking regions are expanded, and the transcription factors, which react with the corresponding DNA regulatory elements, are shown at the bottom. The transcription's initial site is depicted as an arrow at position +1 (Milani *et al.*, 2011)

2.7.1.2. MnSOD enzyme (SOD2)

Manganese superoxide dismutase (MnSOD), Superoxide dismutase 2, and mitochondrial SOD are present in mitochondria that is mediated ROS generated by the partial reduction of O_2. MnSOD act as the first line of defence against reactive oxygen species by catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2).

$$Mn^{3+}+O_2^{\bullet-} \leftrightarrow Mn^{2+}+O_2$$

$$Mn^{2+}+O_2^{\bullet-}+2H^{+} \leftrightarrow Mn^{3+}+H_2O_2$$

Alteration in the function or expression of MnSOD can have significant consequences on mitochondrial function and the general health of cells due to oxidative damage in various mitochondria-localized metabolic processes, leading to the development of different diseases (Hainaut &

Milner, 1993; Miao & Clair, 2009). The *SOD2* gene encodes the MnSOD enzyme on chromosome six which forms a homotetramer and binds one manganese ion per subunit as a cofactor (Perry *et al.*, 2009). The metal is organized by the His26, His74, His163, and Asp159 residue and an oxygen-containing molecule, that can be either water or a hydroxide(Bonetta, 2018). The amino acids layer in the active site of the enzyme is essential to perform the dismutation reaction. The amino acids crucial in the enzyme catalysis mechanism include His30, Tyr34, Phe77, Trp78, Trp123, Gln143, Trp161 and Glu162 from the adjacent subunit(Azadmanesh *et al.*, 2017).(Figure 2.10)

Superoxide anions have proinflammatory roles, causing lipid peroxidation and oxidation, DNA damage, peroxynitrite ion formation and recruitment of neutrophils to sites of inflammation (Droy-Lefaix *et al.*, 1991). Moreover, the SOD2 enzyme is an important enzyme in apoptotic signalling and oxidative stress and plays a vital role in the mitochondrial death pathway, which is required for successful embryonic development and the maintenance of normal tissue homeostasis. (Danial & Korsmeyer, 2004). Numerous researchers have revealed that oxidative stress is significantly increased in women with PCOS (Mohammadi, 2019), and there is an association between Mn superoxide dismutase (SOD2) and PCOS (Azadmanesh & Borgstahl, 2018)

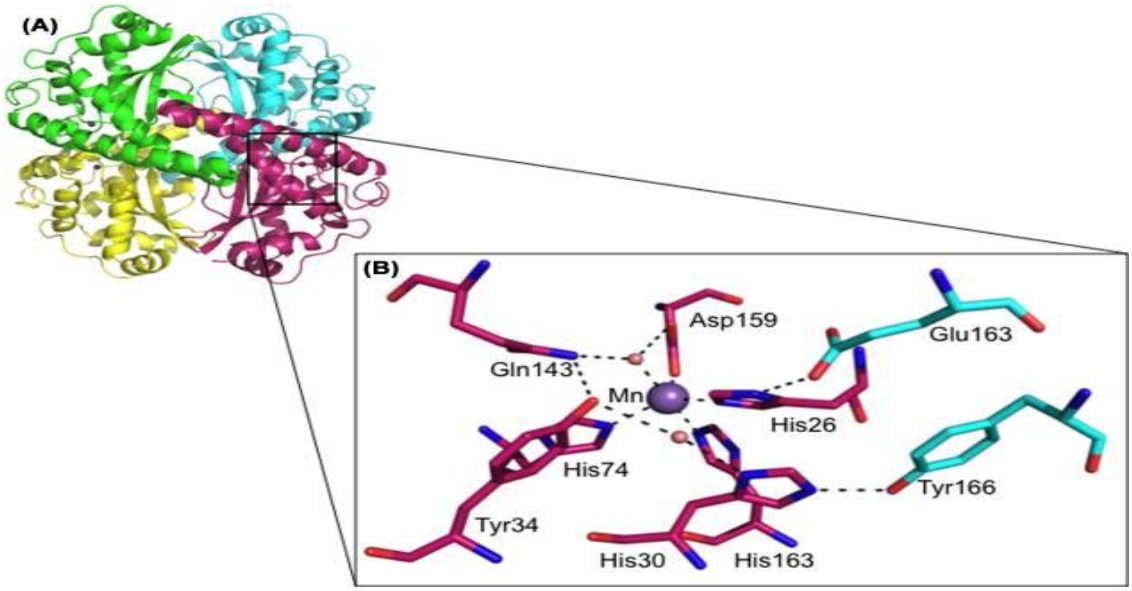


Figure (2.10) : The quaternary and active site structure of human MnSOD.(A) Human MnSOD contains four subunits to form the tetramer. (B) The active site of the human MnSOD enzyme depicts the hydrogen-bonding network from the direction of access to the substrate. (Bonetta, 2018).

2.7.1.2.1. Genetic structures and organization of the *sod2* gene

The human *sod2* is located on chromosome 6q25.3. Based on the molecular structure and organization of the human *sod2* gene, five exons interrupted by 4 introns have been identified. The basal promoter of the *sod2* gene lacks TATA and CAAT boxes but contains GC-rich motifs and numerous Sp1 as well as several AP-2 consensus sequences in its proximal promoter region(WAN et al., 1994).figure(2.11)

A number of studies was identified the association of *sod2* genetic polymorphisms with various diseases including diabetes and hypertension(Nakanishi *et al.,* 2008) Mutations detected in the *sod2* promoter region reveal the possibility for decreased expression of MnSOD in several human cancer cells (Xu *et al*., 1999).

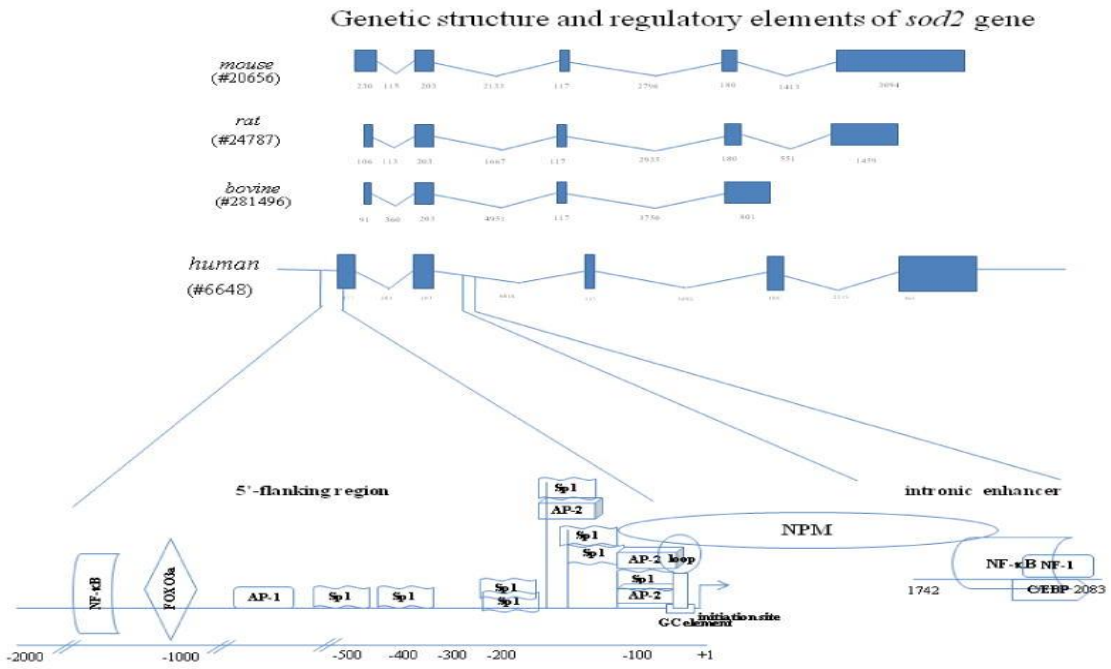


Figure (2.11): Organization of the *sod2* gene, The regulatory elements identified in the 5′ flanking regions and the second intron of the human *sod2* are expanded and shown in the lower part. Corresponding numbers with positive and negative numbers indicate their location relative to the start site, which is designated +1.(Miao & Clair, 2009)

2.7.2. Catalase enzyme

Catalase (EC 1.11.1.6) is one of the most important antioxidant enzymes which detoxify H_2O_2 (A harmful by-product of many normal metabolic processes) into oxygen and water, preventing cellular injury from reactive oxygen species (Wenten M *et al* 2009). Human catalase forms a tetramer that is composed of four subunits, each of which can be divided into four domains. The extensive core of each subunit is generated by an eight-stranded antiparallel β-barrel, A helical domain at one face of the β-barrel is composed of four C-terminal helices and four helices derived from residues between β4 and β5 (Putnam, 2000)**.**

Catalase contains four porphyrin heme or iron groups which allow the enzyme to react with the hydrogen peroxide in which hydrogen peroxide

enters the active site and interacts with the amino acids asparagine and Histidine, causing hydrogen ions to transfer between the oxygen atoms, freeing the newly formed water molecule and Fe(IV)=O. then Fe(IV)=O reacts with a second hydrogen peroxide molecule to reform Fe(III)-E and produce water and oxygen(Karakus, 2020). This enzyme breaks down two hydrogen peroxide molecules into one molecule of oxygen and two molecules of water in a two-step reaction (Nandi *et al.*, 2019) as the following:

$$H_2O_2 + \text{Fe(III)-E} \rightarrow H_2O + \text{O=Fe(IV)-E(.+)}$$
$$H_2O_2 + \text{O=Fe(IV)-E(.+)} \rightarrow H_2O + \text{Fe(III)-E} + O_2$$

The distribution of catalase during different ovarian cycles is related to gonadotropin regulation. Gonadotropins such as FSH have an important function for follicular maturation, differentiation, and steroidogenesis (Fortune J. E.1995).catalase activity was significantly enhanced by gonadotropin stimulation in different mammals. Catalase and estradiol activities in ovarian granulosa cells in different follicle stages were related to FSH levels. Catalase activity increased after FSH stimulation, and the degree of this increase was greater in large follicles than in medium or small follicles; that may suggest a role of catalase in follicle selection and prevention of apoptosis(Parshad & Guraya, 1993).

2.8. Role of SOD genes in PCOS

SOD1 and *SOD2* genes are located on chromosomes 21q22.11 and 6q25.3, expressed in all human tissues (Polat & Şimşek, 2020). it accounts for approximately 50-80% of the total SOD activity and is considered an excellent device against oxidative stress (Weydert & Cullen, 2010).

Insulin resistance has a central role in PCOS. Signs of insulin resistance like hypertension, obesity, metabolic syndrome, nonalcoholic fatty liver, and sleep apnea (Vassilatou, 2014). Most importantly, waist

circumference, independent of body mass index, is responsible for an increase in the oxidation of LDL (Weinbrenner *et al*., 2006). Insulin resistance and hyperinsulinemia increase luteinizing hormone (LH) and the availability of circulating androgen and androgen production by the adrenal gland and ovary mainly by decreasing sex hormone binding globulin (SHBG).(Baptiste *et al.,* 2010). PCOS is associated with decreased antioxidant concentrations and is thus considered an oxidative state (Zuo *et a*l., 2016). The decrease in mitochondrial O_2 consumption, GSH levels, and ROS production explains the mitochondrial dysfunction in PCOS patients(Victor *et al.,* 2011). Increased levels of ROS produced by mononuclear cells during physiological hyperglycemia trigger the release of TNF-alpha and an increase in the inflammatory transcription factor NF-kappa B. TNF-alpha levels, a recognized modulator of insulin resistance, consequently rise even more. As oxidative stress increases, an inflammatory milieu is created, which worsens insulin resistance and causes hyperandrogenism, inhibiting the dominant follicle's growth and preventing apoptosis of small follicles, leading to cyst formation in the ovaries. Hormonal imbalances in females with PCOS are decreased follicle-stimulating hormone (FSH) and FSH/LH ratio, an increase in the level of LH, fasting insulin, estrogen, free testosterone, and a mild increase in prolactin (Rosenfield & Ehrmann, 2016). A lot of studies have exposed that ROS level is significantly increased in patients with PCOS compared to healthy women (Lu *et al*., 2007; Murri *et al*., 2013).

Some studies evaluated gene expression of Cu/Zn SOD and MnSOD in PCOS women in follicular fluid (Seleem *et al.,* 2014) and placental tissue samples and demonstrated increased SODs gene expression. Although both superoxide dismutase enzymes (Cu/zn SOD and Mn SOD) participate in the same metabolic pathway, their locations and transcriptional regulation differ (Zelko *et al*., 2002). *SOD1* expression is

steady, and its products control reactive species turnover in the cytoplasm, allowing cells to maintain homeostasis. SOD2 expression, on the other hand, is sensitive to various internal and external stimuli and serves as the primary defence against oxidative stress within mitochondria.(Williams & Kwon, 2004). Some studies on other women's diseases, such as the (Donabela *et al*., 2015) study, found a significantly higher expression of *SOD1* in women with endometriosis.

2.9. Transcriptional factors involved in the regulation of the *sod* genes

Many transcriptional regulatory elements in the proximal promoter regions of the *sod* genes that are binding sites for several common transcription factors, including NF-κB, AP-1, AP-2, Sp1, and C/EBP, which play essential roles in regulating the constitutive or inductive expression levels of all three SODs**.**

- **NF-κB** acts as a regulator of genes by serving as an immediate response to injurious cellular stimuli. found in both promoter and intronic regions of all three *sod* genes (Oeckinghaus & Ghosh, 2009). Because the *sod1* gene is frequently constitutively expressed and not as easily inducible as other superoxide dismutases, it is considered a (housekeeper gene) and is occasionally used as an internal control to compare variations in MnSOD expression level or activity (Minc E *et al*.,1999)
- **Specificity Protein 1 Sp1** is a zinc-finger protein that acts as a transcription factor by binding directly to DNA through three consecutive zinc-finger domains in the C-terminus and enhances gene transcription (Flashner *et al*., 2022). Multiple GC boxes are the identifiable characteristic of the Sp1-dependent promoter. Thus, the

GC-rich motif contained within the three *sod* gene promoters suggests a common regulatory role of Sp1 in the expression of SODs (Xu Y *et al.*,2002).

- **Activator Protein 1 (AP-1)** AP-1 acts as a transcriptional regulator to modulate signal transduction processes involved in cell proliferation, and transformation (Garces de los Fayos Alonso et al., 2018), the increased DNA binding capacity of AP-1 could cause a reduction in Cu/zn SOD. The activity of AP-1 is topic of redox regulation. Thus, alteration in *sod* genes expression may modulate AP-1 activity (Zhou W *et al*., 2001)
- **Activating Protein 2 (AP-2)** AP-2 is a family of closely related transcription factors consisting of AP-2alpha, AP-2beta, AP-2gamma, AP-2delta and AP-2epsilon (Eckert D *et al.*,2005). In addition to direct binding to the target gene, it can crosstalk with other transcriptional factors to alter the expression of a specific gene (Xu Y *et al.*,2008**).** On the other hand, AP-2 plays a negative role in the constitutively low expression of MnSOD by suppressing Sp1-dependent transcription (Zhu CH *et al.*,2001) **.**
- **CCAAT-Enhancer-Binding Proteins (C/EBP)** C/EBP proteins consist of six members, C/EBP α to C/EBP ζ, which can interact with the CCAAT box motif present in many gene promoters. C/EBP- factors are necessary for *sod1* transcription (Ramji & Foka, 2002)
- **FOXO** The family of forkhead box class O (FoxO) transcription factors includes FoxO1, FoxO3, FoxO4, and FoxO6. FoxOs regulate genes involved in various pathways such as metabolic regulation, cell and tissue homeostasis, and immunity (Calissi et al., 2021).Foxo characterized by a winged helix DNA binding domain known as a

Forkhead box.FOXO transcription factors function mostly as transcriptional activators, and their activity is inhibited by insulin and growth factor signalling (Kaestner *et al*., 2000). During oxidative stress, FoxO4 binds to the promoter of the *SOD2* gene and induces the expression of manganese superoxide dismutase, an antioxidant enzyme located within the mitochondrial matrix (Araujo *et al*., 2011).

3. Materials and Methods

3.1. Materials

3.1.1 . Equipment

Table (3.1) Equipment and apparatus used in this study

Device	Company	Origin
Cooling centrifuge	Hittch	Germany
Laminar flow UV cabinet	Labtech	Korea
Vortex Mixer	Witeg	Germany
Centrifuge	Fisher Scientific	USA
Mic qPCR Cycler	Bio Molecular System	Australia
Mic Tube	Bio Molecular System	Australia
Quantus Fluorometer	Promega	USA

3.1.2. Chemicals

Table (3.2): Chemical materials used in this study.

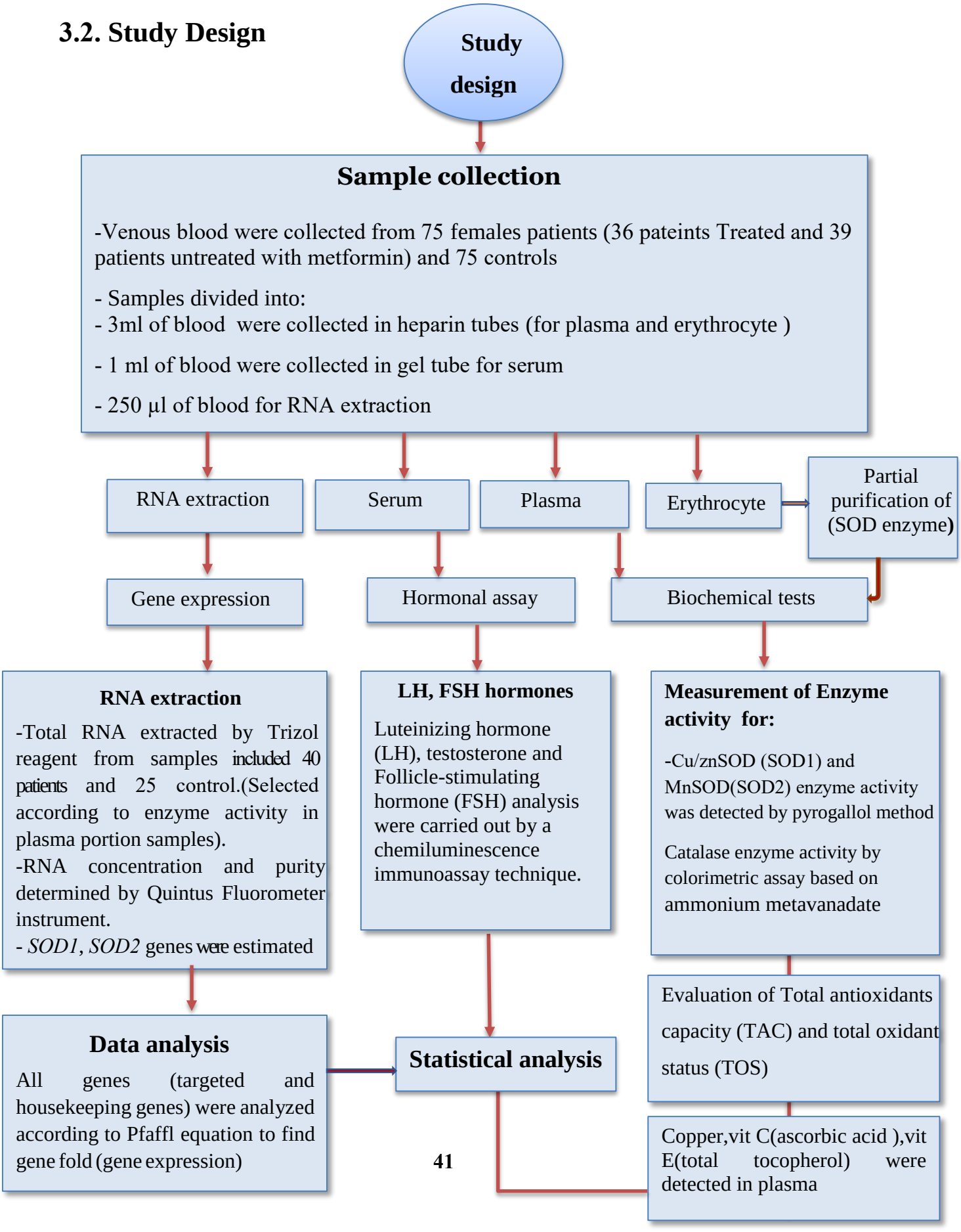
Chemical materials	Manufacture	Origin
Isopropanol	High Media	India
TRIzol Reagent	Thermo fisher Scientific	USA
Chloroform	LiChrosolv	Germany
Primers	Macrogen	Korea
GoTaq® 1-Step RT-qPCR System, MgCL2, Nuclease Free Water, Quantifluor RNA System	Promega	USA

3.1.3. Primers

Table (3.3): Primers of *SOD1*, *SOD2* gene and *β-actin*(housekeeping gene) used in this study

Primer name	Sequence 5` ⟶ 3`	Annealing Temp.(C)	References
***SOD1*-F**	**CGAGCAGAAGGAAAGTAATG**	**52**	**(Sugino *et al.*, 2000)**
***SOD1*-R**	**TAGCAGGATAACAGATGAGT**		
***SOD2*-F**	**AGTTCAATGGTGGTGGTCATA**	**60**	
***SOD2*-R**	**CAATCCCCAGCAGTGGAATAA**		
***β-actin*-F**	**CCCTGGACTTCGAGCAAGAG**	**62**	**(Rong *et al.*, 2016)**
***β-actin*-R**	**TCACACTTCATGATGGAGTTG**		

3.2. Study Design



3.3. Subjects

A total number of 75 females with PCOS were involved in this study. samples were collected during the period from November 2019 until October 2021, the samples were collected from females (married and unmarried/ patients and controls) who regularly visited Imam AL-Sadeq hospital and private gynaecology clinic in AL- Hillah city whose ages ranged from (20-45) years, Metformin treatment were included in this study in which PCOS women divided into two groups: PCOS patients treated with metformin (36 patients)and PCOS patients untreated with metformin (39 patients). (Participants were provided with an appropriate understanding of the research work and written informed agreement was provided by participants before collecting samples or any data. Related information about the participants', socio-demographic and health history were collected by using questionnaires.). Physicians evaluated the clinical assessment of patients with PCOS according to sonograph and laboratory assessment; hormone levels FSH, LH were estimated. The questionnaire form has been filled out for each patient and control as shown in appendix (A).

3.3.1. The inclusion criteria used for the recruitment of PCOS subjects

PCOS was diagnosed according to Rotterdam criteria (Fauser *et al.*, 2012). Two out of three of the following criteria were met for the diagnosis:

1. Clinical and biochemical signs of hyperandrogenism.

Clinical: Hirsutism was considered the indicator of hyperandrogenism. Modified Ferriman-Gallwey (MFG) score test was taken as a sign of locations in the body, The MFG score evaluates hair growth in nine androgen-sensitive body areas: the upper lip, chin, chest, upper and lower back, upper

and lower abdomen, upper arm, and thigh. Hair distribution score ≥8 of 36 (Ilagan *et al.*, 2019) (Figure 3.1). **Biochemical:** Androgen values greater than the reference range (total testosterone and dehydroepiandrosterone sulfate (DHEA-S)) were determined as hyperandrogenemia.

2. Oligo ovulation or non-ovulation; Cycle ranged from 35-45 days (oligomenorrhoea) or absence of menstruation for more than 3 months (amenorrhoea).

3. Appearance of polycystic ovaries on ultrasound. In 3rd or 4th day of the menstrual cycle, ultrasonic evaluation was performed with transvaginal ultrasound to check the morphological appearance of the ovaries.

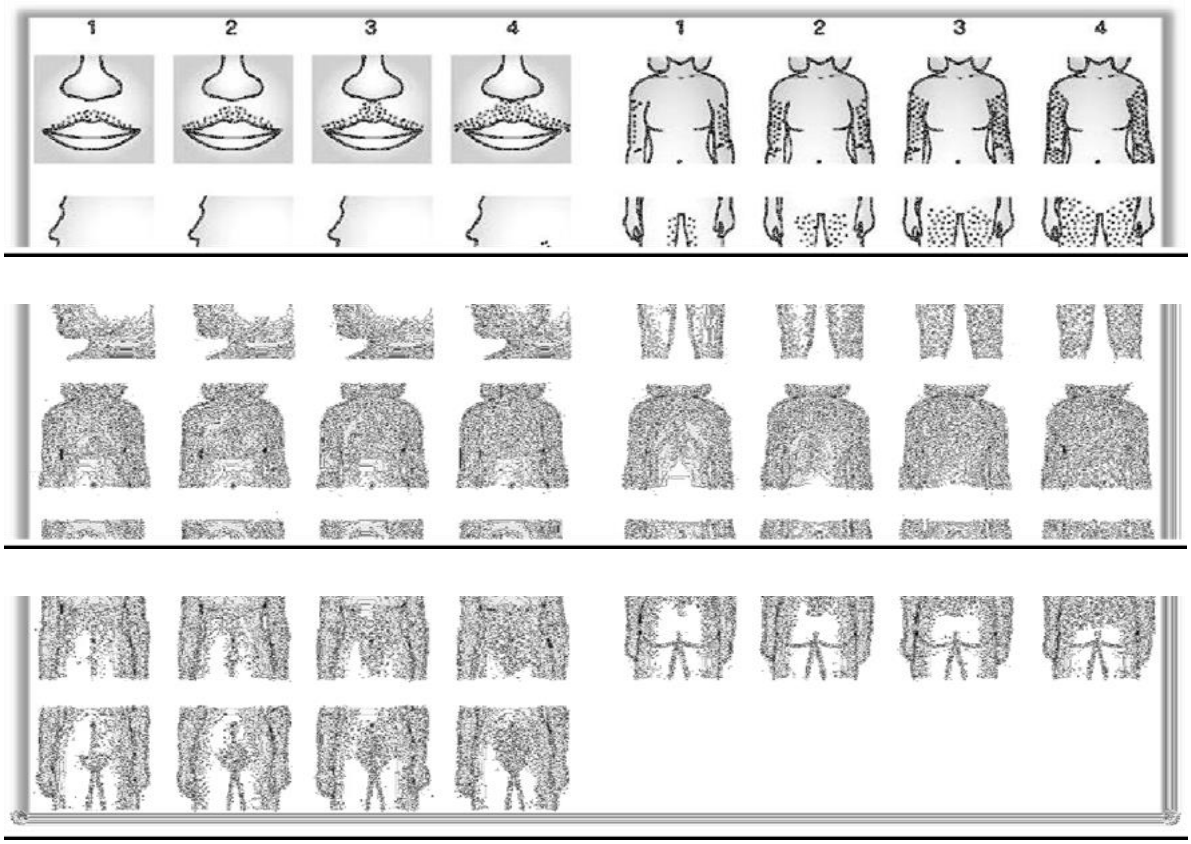


Figure (3.1) : Ferrimane Gallwey hirsutism scoring system (Hatch et *al.*, 1981).

3.3.2. The inclusion criteria used for the recruitment of the control group

1. The length of their menstrual cycles ranged between 22-35days.
2. History of spontaneous conception.
3. They had no clinical or biochemical signs of hyperandrogenism and no polycystic ovaries at any stage of life.
4. Body mass index (BMI) for both patients and the healthy control group was measured as follows: weight (kilogram), Height (m2).

3.3.3. Disorders that possibly mimic PCOS and are excluded from the Rotterdam criteria:

1. Hyperprolactinemia is an increase in prolactin levels above the reference range. Although elevated prolactin levels have been reported in women with PCOS, hyperprolactinemia and PCOS should be treated as two separate clinical entities (Davoudi *et al.*, 2021).

2. Thyroid disease, as measured by levels of thyroid-stimulating hormone (TSH), subjects with elevated or low TSH were excluded from this study.

3. Non-classical congenital adrenal hyperplasia, was excluded from the study.

4. women taking contraceptives or induction, **smoked women, or having hypertension or diabetes,** are all excluded.

3.4. Body Mass Index (BMI)

Body mass index (BMI) is commonly used as a substitute marker and indicates weight-for-height without considering differences in body composition and the contribution of body fat to overall body weight (Pasco *et al*., 2014) BMI (kg/m^2) = weight (kg) / $(height)^2$ $(m)^2$

There are four BMI categories:

1. BMI of fewer than 18.5 is considered underweight.

2. BMI values between 18.5 and 24.9 are considered a normal or healthy weight.

3. BMI values between 25 and 29.9 are considered overweight.

4. BMI 30 and above are considered obese (BMI 30-39.9 severely obese, BMI 40-49.9 morbidly obese, and BMI>50 represents super obese).

3.5. Collection of Blood and Sample Preparation

Five ml of blood was obtained from each participant by vein puncture, The blood sample was obtained during the early follicular phase of a menstrual cycle (days 2–5). Blood samples were collected in heparin tubes (for plasma and erythrocyte, approximately 2 ml of blood), 2 ml of blood in a gel tube for serum separation, and 250 microliters of blood with 750 microliters of trizol for RNA extraction.

3.5.1. Preparation of erythrocyte

Blood samples were centrifuged at 1000 × g for 10 min at 4˚C to remove the plasma. The buffy coat on the erythrocyte sediment was separated carefully after the plasma was removed. The plasma portion was frozen at -20˚C until the time of analysis. Erythrocytes (packed cells) were washed 3 times by centrifugation with 3 volumes of cold 0.15 M NaCl (0.9-gram NaCl in 100 ml DW.) at 4000 g for 5 min. The cells were frozen at -20 ˚C and stored or lysed by thawing. The hemolysate was diluted by adding 3 volumes of ice-cold deionized water. The temperature was maintained at 0-2 ˚C by using an ice water mixture while ethanol was added with adequate stirring to a final concentration of 25% (v/v). Cold chloroform was added to the mixture to a concentration of 12% (v/v). Stirring was continued for 30 minutes, during

which the haemoglobin was rendered insoluble, and centrifuged for 10 minutes at 3000 rpm. The enzyme is contained in the clear top layer. The resulting supernatant was allowed to warm at room temperature, and solid K_2HPO_4 (300g/l) was added, separating two liquid phases. The denser phase was essentially aqueous and contained most of the salts, the lighter phase was ethanol and contained SOD and little salts. The upper phase was collected and centrifuged. The supernatant contained all of the SOD activity(Djalali *et al.*, 2005).

3.6. Superoxide Dismutase (SOD) enzyme activity

3.6.1. Principle

(Cu\Zn) SOD enzyme activity was determined by used a simple and rapid method based on the ability of the enzyme to inhibit the autoxidation of pyrogallol. The autoxidation of pyrogallol in the presence of EDTA in pH (8.2) is 50%. The Principle of this method is based on the competition between pyrogallol autoxidation by $O_2\bullet^-$ and the dismutation of this radical by SOD(Marklund & Marklund, 1974).

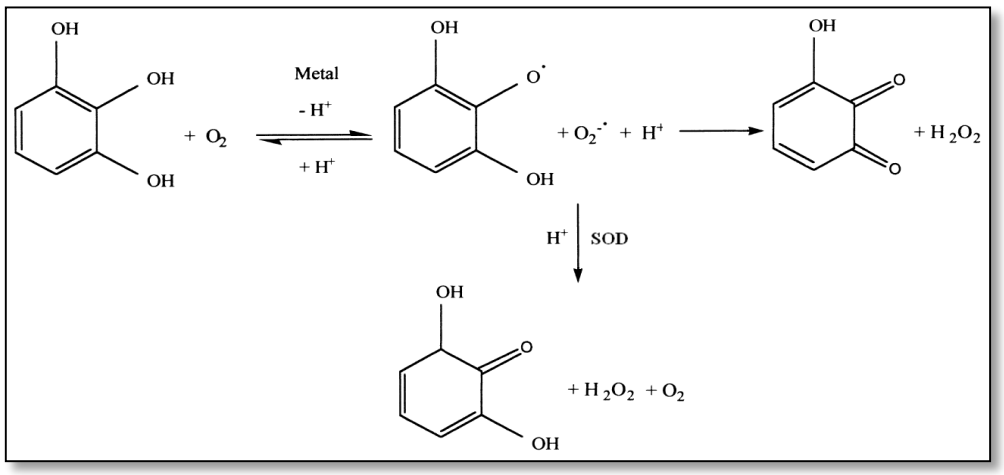


Figure (3.2): Suggested Mechanism of Pyrogallol Autoxidation.

(Cu\Zn) SOD activity is expressed as units/ml. One unit of (Cu/Zn) SOD activity is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation.

3.6.2.Reagents Preparation

1. Tris- EDTA buffer pH 8.2 A weight of 2.85 g of Tris and 1.11 g of EDTA-Na_2 were dissolved in 1 liter of DW. Adjusted with 0.1 M HCl into pH 8.2.

2. Pyrogallol Solution (0.2 mM) A weight of 0.252 g of pyrogallol was dissolved in a solution of 0.6 ml of concentrated hydrochloric acid diluted in 1 liter of DW

3.6.3. Procedure

A spectrophotometer was adjusted to read zero using a Tris-EDTA buffer. Control and sample test tubes were prepared and then pipetted into test tubes

Reagents	Test (µl)	Control (µl)
Sample	50	-
Tris-buffer	1000	1000
DW	-	50
Pyrogallol	1000	1000

Absorption read at the wavelength of 420 nm against Tris-EDTA buffer at zero time and after 1 minute and after 2 minutes of the pyrogallol addition.

3.6.4. Calculation of SOD activity:

$$\% \text{ Inhibition of pyrogallol autoxidation} = \frac{\Delta A \text{ CONTROL} - \Delta A \text{ test}}{\Delta A \text{ control}} \times 100\%$$

$$\text{SOD Activity (U/ml)} = \frac{\text{inhibition of pyrogallol autoxidation}}{50\%} \times \%$$

*ΔA = Absorption after 2 min – Absorption at zero time

3.7. Manganese Superoxide Dismutase (MnSOD) enzyme activity

3.7.1. Principle

MnSOD enzyme activity was determined according to the method described by (Marklund and Marklund,1974; and Del Maestro *et al.*,1983), which is based on the ability of MnSOD to inhibit the auto-oxidation of pyrogallol in the presence of sodium cyanide to determine SOD activity in the sample.

3.7.2. The reaction mixture:

The reaction mixture consisted of

1. Tris- EDTA buffer pH 8.2
2. Pyrogallol Solution (0.2 mM)
3. 0.1 M NaCN (1 liter of Assay buffer containing 4.901gm NaCN)
4. Sample.

Cu\Zn SOD activity was inhibited by adding (1000 µl) of assay buffer containing NaCN for 15 min; the reaction was initiated by adding (1000 µl) of pyrogallol (final concentration of 0.2 mM), and the absorbance was measured at 420 nm. One unit of activity is defined as the amount of sample needed to inhibit pyrogallol oxidation by 50 %. The final results were expressed as U/ml(Stojkovski *et al.*, 2013).

3.7.3. Calculation of SOD activity:

$$\% \text{ Inhibition of pyrogallol autoxidation} = \frac{\Delta A \text{ CONTROL} - \Delta A \text{ test}}{\Delta A \text{ control}} \times 100\%$$

$$\text{SOD Activity (U/ml)} = \frac{\text{inhibition of pyrogallol autoxidation}}{50\%} \times \%$$

*ΔA = Absorption after 2 min – Absorption at zero time

3.8. Catalase enzyme activity

3.8.1. Principle

Catalase enzyme activity in biological fluids and tissues was determined by a colourimetric assay in which H_2O_2 dissociation rates are directly proportional to catalase activity. The Principle of this procedure was based on reactions of ammonium metavanadate with H_2O_2 under acidic conditions. The resulting reduction of vanadium (V) to vanadium (III) by H_2O_2 produces a red-orange peroxovanadium complex. The reaction between vanadium and H_2O_2 is shown in the following equation:

$$H_2O_2 + NH_4VO_3 + H_2SO_4 \rightarrow NH_4[VO(O_2)SO_4] + 2H_2O$$

Catalase enzyme activity was determined by monitoring the absorption of the red-orange peroxovanadium complex at 452 nm (Hadwan & kadhum Ali, 2018).

3.8.2. Reagents preparation

1. Sulfuric acid solution (0.5 M) was prepared by appropriate dilution of concentrated sulfuric acid in 200 ml of distilled water.

2. Ammonium metavanadate solution (0.01 M) contained 0.2925 g of ammonium metavanadate in 200 ml of 0.5M sulfuric acid.

3. Phosphate buffer (50 mM; pH 7.0) was prepared by mixing solutions (a) and (b) at a ratio of 1:1.5 a:b. **solution (a)** was prepared by dissolving 6.81 g of KH_2PO_4 in one liter of distilled water, and **solution (b)** was prepared by dissolving 8.90 g of $Na_2HPO_4.2H_2O$ in one liter of distilled water.

4. Fresh H_2O_2 (10 mM) solutions were prepared by mixing 0.1134 ml of 30% H_2O_2 with 100 ml of phosphate buffer, and the solution was adjusted to 10mM by using the molar extinction coefficient of H_2O_2 at 240 nm (43.6 $M^{-1}cm^{-1}$).

3.8.3. Procedure

Following (table 3.4) is the procedure for catalase activity analysis.

Table (3.4): Procedure for assessments of catalase activity.

Reagents	Test	Standard	Blank
Sample	100 µl	------	-----
Phosphate buffer	900 µl	1000 µl	3000 µl
Hydrogen peroxide	2000 µl	2000 µl	----
Mix with vortex and incubate at 37 °C for 2 min; after that, add:			
Vanadium reagent	2000 µl	2000 µl	2000 µl
After that, the tubes were kept at 25 °C for 10 min. The changes in absorbance were recorded at 452 nm against the blank.			

3.8.4. Calculation

The enzyme activity procedure was elucidated in (Table 1). The rate constant (k) of the first-order reaction equation for catalase activity was calculated using the following formula:

$$\text{Catalase Activity of test kU} = \frac{2.303}{t} * \frac{\log S^o}{S}$$

where t is time, S^o is the absorbance of the standard solution, and, S is the absorbance of the sample, and kU (katal unite)

3.9 Determination of Total Oxidant Status (TOS).

3.9.1. Principle

TOS of the sample was measured by using a method developed by Erel O 2005. Oxidants in the plasma oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is improved by glycerol molecules abundantly found in the reaction medium. The ferric ion creates a colored complex with xylenol orange in an acidic medium. Color intensity, which can

be determined spectrophotometrically, is associated with the sample's total amount of oxidant molecules. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 Eq/L) (Erel, 2005).

3.9.2. Reagents

Reagent 1: was prepared by dissolving 1.96 g of ferrous ammonium sulfate and 3.17 g of O-dianisidine dihydrochloride in 1000 mL of 25 mM H_2SO_4 solution. The final reagent consisted of 5 mM ferrous ammonium sulfate and 10 mM O-dianisidine dihydrochloride.

Reagent 2: was prepared by dissolving 114 mg of xylenol orange and 8.18 g of NaCl in 900 mL of 25 mM H_2SO_4 solution. One hundred milliliters of glycerol was added to the solution. The final reagent was composed of 150 µM xylenol orange, 140 mM NaCl and 1.35 M glycerol. The pH value of the reagent was 1.7. This reagent is stable for at least 6 months at 4°C.

Hydrogen Peroxide (STD): (100 µmol/L) was freshly diluted and standardized daily by using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm .

3.9.3. Procedure: Following (table 3.5) is the procedure for TOS analysis.

Table (3.5): Shows the Details of the TOS analysis method.

	Blank	Standard	Sample
Distilled water	100 µl	-----	------
Sample	------	-----	100 µl
Hydrogen peroxide	------	100 µl	------
R1	1 ml	1 ml	1 ml
Test tubes were mixed by vortex, then added:			
R2	0.25 ml	0.25 ml	0.25 ml
Gently mix the content of every tube after addition, let to stand at room temperature for 30 minutes, read spectrophotometrically at 560 nm.			

3.9.4. Calculation

$$\text{Total oxidants status} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD$$

3.10.Total Antioxidants Capacity Assay (TAC): The CUPRAC method.

3.10.1. Principle

The CUPRAC assay estimates the capacity of the antioxidants in samples to reduce the Cu^{2+} to Cu^{1+} in the presence of a chelating agent. These chelators form a stable color complex with Cu^{1+} that have absorption at 450 nm. The CUPRAC assay measures thiol-group antioxidants and other plasma antioxidants such as ascorbic acid, α-tocopherol, β-carotene, uric acid, albumin, and bilirubin.

Antioxidants + Cu^{+2} ⟶ Cu^{+1}

Cu^{+1} + 2, 9-dimethyl-1, 10-phenanthroline ⟶ complex (λ_{max} at 450 nm)

(Apak *et al.*, 2008).

3.10.2.Reagents

1. Copper(II) chloride solution at a concentration of 10^{-2}M was prepared by dissolving 0.4262 g of $CuCl_2 .2\ H_2O$ in 250 ml of distilled H_2O.

2. Ammonium acetate (NH_4Ac) buffer pH = 7.0 was prepared by dissolving 19.27 g of NH_4Ac in water and completing the volume up to 250 ml.

3. Neocuproine (Nc) solution at a concentration of $7.5x\ 10^{-3}$M was prepared by dissolving 0.039 g Nc in 96% EtOH, and the volume was completed to 25 ml with ethanol.

4. The standard solutions of sample antioxidants were prepared at 1.0×10^{-3} concentration of Trolox.

3.10.3.Procedure

Following (table 3.6) is the procedure TAC analysis by the **CUPRRAC method**.

Table (3.6) Shows the Details of the CUPRRAC method.

Reagents	Test	Standard	Blank
Copper(II) chloride solution	1ml	1ml	1ml
Sample	50 µl	------	-------
Working standard solution	-------	50 µl	-------
D.W	-------	-------	50 µl
Neocuproine (Nc) solution	1ml	1ml	1ml
Ammonium acetate (NH_4Ac) buffer	1ml	1ml	1ml

3.10.4.Calculation

$$\text{Total antioxidants levels} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD\left(\mu mol/l\right)$$

3.11. Measurement of free copper by (LTA Kit)

3.11.1. Principle

In the copper test, chromogen 3,5-Di-Br-PAESA was reacted with cupric ions and formed a blue-violet compound; the color intensity is proportional to the copper concentration in the sample

3.11.2. Reagents

Reagent A: Acetate buffer 0.1M, PH 4.9 (prevents the pH of a solution from changing drastically)

Reagent B : 3,5-Di-Br-PAESA

Standard: Ion copper 200 µg/dl

3.11.3. Procedure

A. Determination of copper in plasma: Reagent preparation includes preparing of working reagent by mixing an equal quantity of Reagent A with Reagent B, which remains stable for 20 days at room temperature.

Table (3.7): procedure for copper determination in plasma.

Reagents	Test	Standard	Blank
Work reagents	1ml	1ml	1ml
Distilled water	66µl	…..	……..
Standard	……..	66µl	…….
Plasma	……..	……	66µl

Mix and wait for 10 min, then read the absorbance against the blank at 580 nm.

B. Calculations

Copper µg/dl = A(Sample)\ A (Standard) * 200

3.11.4. Normal values

Man	**80- 140 µg/dl**
Woman	**80-155 µg /dl**

3.12. Assay of total tocopherol (vitamin E) in plasma

3.12.1. Principle

Plasma total tocopherol was assayed by the method of (Quaife *et al.*, 1949), It involves the Emmerie- Engel color reaction with ferric chloride and α,α-dipyridyl to give a red color

3.12.2. Reagents:

1. absolute ethanol.

2.α,α-dipyridyl: was prepared by dissolving 0.120 gm of α,α-dipyridyl in 100 ml of n-propyl alcohol.

3. Ferric chloride hexahydrate: was prepared by dissolving 0.120 gm of Ferric chloride hexahydrate in 100 ml of absolute ethanol. This solution was kept in a dark brown or red glass bottle.

4.α-tocopherol standard (1µmol/L) was prepared by dissolving 2.0 mg of α-tocopherol in 100 ml of absolute ethanol.

3.12.3. Procedure:

Following the procedure of vit E determination in plasma.

Table (3.8): procedure of vit E determination in plasma.

Reagents	Test	Standard	Blank
Absolute ethanol	0.6 ml	0.6 ml	0.6 ml
Sample	0.6 ml	------	-------
D.W.	-----	------	0.6 ml
STD	-----	0.6 ml	-------

Xylene	0.6 ml	0.6 ml	0.6 ml
Mixed well and centrifuged for 10 min at 3000 rpm.			
xylene supernatant layer	0.4 ml	0.4 ml	0.4 ml
α,α'-dipyridyl was added and vortexed	0.4 ml	0.4 ml	0.4 ml
The 0.6 ml of this mixture was pipetted into a cuvette and the absorption was measured spectrophotometrically at 460 nm against deionized water.			
ferric chloride	0.13 ml	0.13 ml	0.13 ml
Mixed thoroughly and absorption was again read at 520 nm spectrophotometrically exactly 1.5 min after the addition of ferric chloride.			

3.12.4. Calculation

$$\text{Conc. of test} = \frac{(\text{A } 520 - 0.29\,\text{A } 460)\ \text{test}}{\text{A } 520\ \text{STD}} * \text{Conc. of STD}$$

3.13. Determination of the Total Vitamin C (Ascorbic Acid) in plasma

3.13.1. Principle

In the 2,4-dinitrophenylhydrazine (DNPH) methods, Ascorbic acide is oxidized by Cu+2 to DHA (dehydroascorbic acid) and diketogulonic acid (Burtis, 1999). When treated with DNPH (Dinitrophenyl hydrazine), the 2,4-dehydrophenylosazon product forms, which, in the presence of sulfuric acid, forms an orange-red complex that absorbs at 520 nm as shown in (figure 3.3

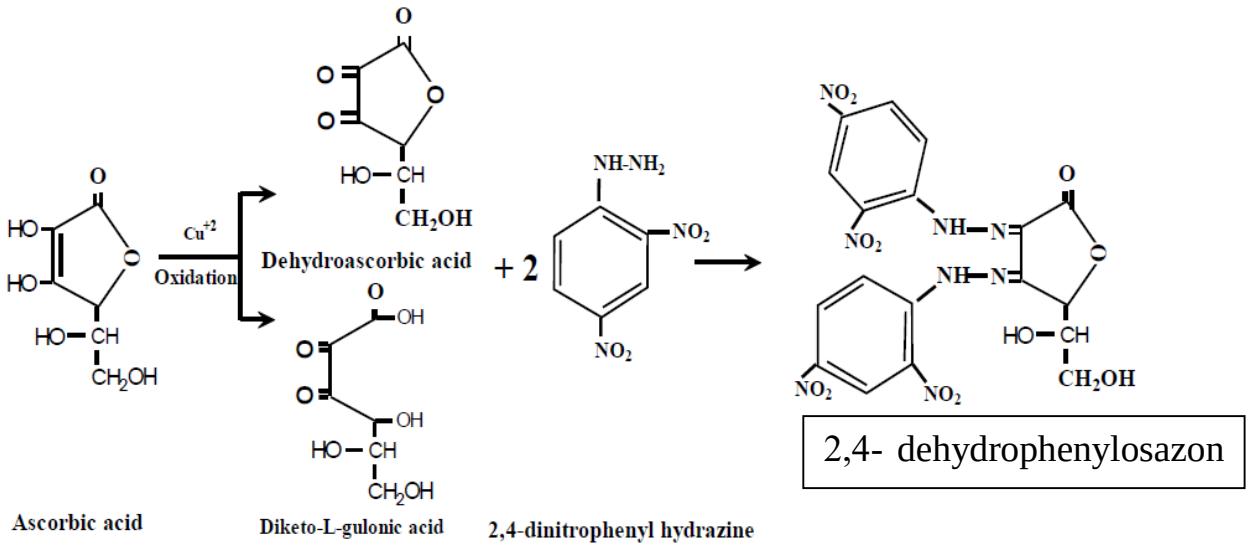


Figure (3.3): Reaction Between Ascorbic Acid and 2,4-DNPH (Lloyd *et al.*, 1945).

3.13.2. Preparation of Reagents

1. Metaphosphoric acid (m-HPO3) (0.75M) 30gm of m-HPO3 are dissolved in a final volume of 500 ml ddH_2O. (Stable for 1 week).

2. Sulfuric acid H2SO4 (4.5M) Carefully 250 ml of concentrated H2 SO4 are added to 500 ml of cold ddH_2O. When the solution has been cooled at room temperature, ddH_2O is added up to 1 liter, with mixing (Stable for 2 years).

3.Sulfuric acid H2SO4 (12M) Carefully 650 ml of concentrated H_2SO_4 are added to 300 ml of cold ddH_2O and brings to a final volume of 1 liter (Stable for 2 years).

4. 2,4-DNPH (2,4-Dinitrophenyl hydrazine) reagent (0.01M) 10 gm of 2,4-DNPH were dissolved in 4.5 M H_2SO_4 and bring to a final volume of 500

ml, then refrigerated overnight, and filtered. (Stable for at least 1 week at refrigerated temperature).

5. Thiourea (0.66M) 5 gm of thiourea are dissolved in a final volume of 100 ml of ddH_2O. (Stable for 1 month at 4C°).

6. Copper sulfate (0.027M) 0.6 gm of anhydrous copper sulfate is dissolved in a final volume of 100 ml of ddH_2O. (Stable for 1 year at room temperature).

7. DTCS reagent: 100 ml of the 2,4-DNPH reagent, 5 ml of the thiourea,and 5 ml of the copper sulfate are combined. (Store in bottle at 4C° for a maximum of 1 week).

8. Ascorbic acid standards Stock standard solution (2.8 mM) is prepared by dissolving 50 mg of ascorbic acid in a final volume of 100 ml of m-HPO_3. Dilutions are made by m-HPO_3 to 2.5, 5 and 20 mg/L (0.014, 0.028, and 0.11 mM) respectively. There are the working standards (All working standards should be prepared freshly).

3.13.3. Procedure

The procedure for determination of total vitamin C in plasma by 2,4-DNPH method is summarized as follows: Each standard and sample test tube are prepared, then pipetted into test tubes

Reagents	Sample(µL)
m-HPO3	800
plasma	200

Tubes are mixed in vortex mixture, then centrifuged at 2500 x g for 10 minutes

Reagents	Sample(µL)	Reagent Blank(µL)	Standard(µL)
Supernatant	600		
Standards			600
m-HPO3		600	
DTCS reagent	200	200	200

Tubes are capped and mixed in a vortex mixture, then incubated in a water bath at 37C° for 3 hours.

The tubes are removed from the water bath and chilled for 10 minutes in an ice bath, with slowly mixed

Reagents	Sample(µL)	Reagent Blank(µL)	Standard(µL)
Cold H2SO4 (12M)	1000	1000	1000

Tubes are mixed in a vortex mixture and returned immediately to the ice bath. The spectrophotometer is adjusted with blank to read zero absorbance (A) at 520 nm, and the absorbance of standards and sample is read.

3.13.4. Calculation of plasma Vitamin C

The concentrations of the samples were obtained from the calibration curve (Figure 3.4) and were multiplied by 5 to normalize the dilution of the plasma m- HPO3) to give the concentration of vitamin C (ascorbic acid) per liter of sample. The concentration of ascorbic acid can be determined directly from a standard as follows:

$$\frac{\text{A sample}}{\text{C sample}} = \frac{\text{Astd}}{\text{C std}}$$

$$\text{C sample} = \frac{\text{C std}}{\text{A std}} * \text{A sample} * 5$$

where: Astd and Cstd

C = concentration mg/L of AA in sample and standard.(std)

A = absorbance at 520 nm for sample and standard.

5 = factor is added to normalize the dilution of samples.

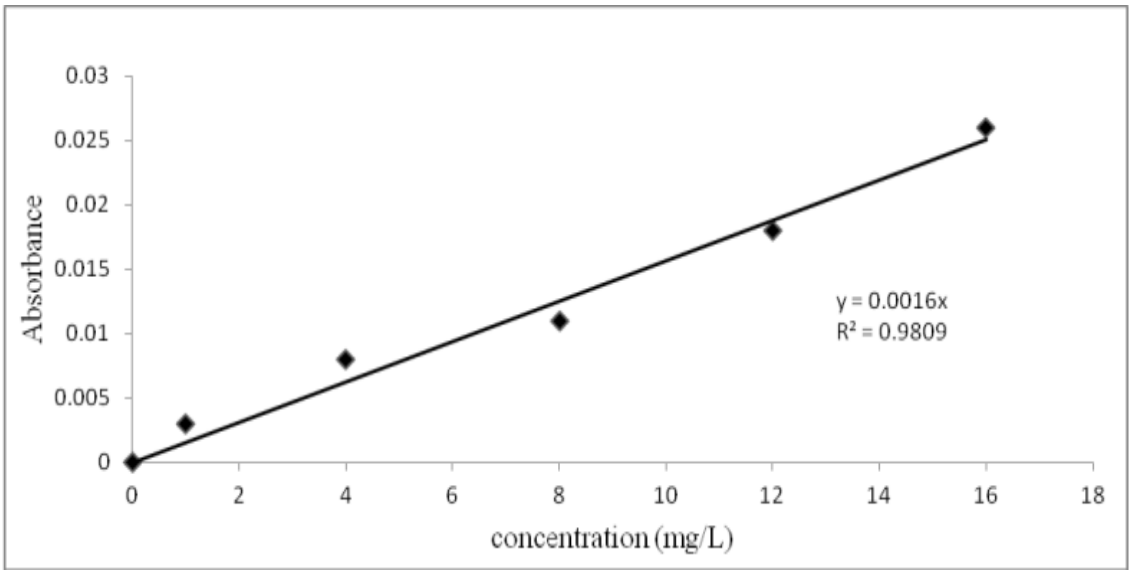


Figure (3.4): Standard curve of ascorbic acid

3.14. Determination of serum Hormones

3.14.1. Determination of Luteinizing Hormone (LH) by using MAGLUMI LH (CLIA) Kit

٣.14.1.1 Principle

Method is used an anti-LH monoclonal antibody to label *N*-(4-aminobutyl)-*N*-ethyl-isoluminol (ABEI), and used another monoclonal antibody to coat magnetic microbeads. Sample, Calibrator or Control, with ABEI Label and magnetic microbeads are mixed thoroughly and incubated at 37℃, forming a sandwich; After sediment in a magnetic field, suck the supernatant and then cycle wash it for one time. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as as Relative Light Units (RLU) within 3 seconds and is proportional to the concentration of LH present in samples

3.14.1.2. Reagents integral preparation

1. Microbeads were mixed prior to loading the reagent assembly onto the system for the first time to resuspend the microbeads that had settled during shipment.
2. The microwheel in the microbead chamber was rotated back and forth until the color of the suspension changed to brown, waited for 30 minutes for the integrator to sit in the reagent compartment. The magnetic microbeads are automatically agitated and completely suspend during this period
3. The solutions were stored at 2-8 °C before use and are good until the expiration date. The solutions are stable for 30 days after they are

opened. When not in use, open solutions should be stored in the refrigerator for the greatest results

3.14.1.3. Procedure

1. The device reagents have been downloaded after scanning the device space automatically in the device test, the group has been suspended on the bead in the automatic confusion solution that occurs after loading for 30 minutes and then the detector and sample as well as to increase stability.

2. The volume of 40µL sample has been added, the control tube, the intense location, and the device has been entered, and the beginning was specified.

3. The sample was mixed with maglumi system ABEI label + 80µl, nanomagnetic microbeads + 20µl then incubated for 15-minute at 37 ° C.

4. Cuvette was transferred to washing station to a cycle of washing for 3 times to remove the illegal content by 400 µl washing buffer (one bottle of concentrated for concentrated washing to 10 liters for this purpose.

5. Cuvette has been moved to a triangular measurement captured by the room that is a dark room, here, 2 substrates. Starter 1+ 2 Then is run with the Current Chemiluminescence label: ABEI, the light signal is measured by a photomultiplier within 3 seconds

6. After finished measured, Cuvette has been paid to the waste bag and the end of the test.

3.14.1.4. Calculations

The starter detector has been added to start the chemiluminescent interaction, and the production of a light signal is measured by photomultiplier, which suit the LH concentration in the sample, the analyzer automatically calculates LH concentration in each sample through the calibration curve created by the calibration curve 3 points. The results are expressed in mIU / ml.

3.14.2. Determination of serum Follicle-stimulating hormone (FSH) concentration by using MAGLUMI LH (CLIA) Kit

3.14.2.1. Principle

Sandwich immunoluminometric assay was used an anti-FSH monoclonal antibody to N-(4-aminobutyl)-N-ethyl-isoluminol (ABEI) label, and has been used another monoclonal antibody to coat magnetic microbeads. Sample, Calibrator or Control, with ABEI Label and magnetic microbeads are mixed thoroughly and incubated at 37℃, forming a sandwich; After sediment in a magnetic field, suck the supernatant and then cycle wash it for 1 time. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as Relative Light Units (RLU) within 3 seconds and is proportional to the concentration of FSH present in samples

3.14.2.3. Procedure

1. The reagents were loaded into the device after scanning the code using the automatic area scanning of the device in order to take the test device, the group containing the bead was suspended in the solution via the automatic mixing that occurs after loading for 30 minutes, and then the reagent and sample area was cooled to increase stability.

2. A volume of 40 μ L of sample and control was placed in a collection tube, entered the site and condenser device, and then the starting was determined.

3. Sample needle and reagent sampler were taken into a cuvette; serum was combined with AbEI Label + 80 μ L and Nanomagnetic Microbeads + 20 μ L from the Maglumi System kit.

4. Mixture was incubated at 37 °C for 15 minutes.

5. The cuvette was transferred to the washing station and washed cycle washed three times with 400 L wash buffer to remove non-agglutinating contents (concentrated wash contents are diluted to 10 L for this purpose).

6. The cuvette was moved to a three-second fitting room, which is a dark room, and two braces were used, starters 1 and 2 were applied, and they reacted with the mark of ABEI Chemiluminescence.

7. The cuvette was pushed into the garbage bag after being measured, and the test was completed.

3.14.2.4. Calculation Result

The initiator reagent was added to start a luminous chemical reaction, which resulted in an optical signal measured in a photomultiplier as relative luminous units (RLUs) which are proportional to the concentration of FSH present in the sample, and the analyzer automatically calculated the FSH concentration in each sample by means of the generated calibration curve By performing a two-point master calibration curve. The results were expressed in mIU/ml,The sample concentrations were between 0.02-78.92mIU/ml.

3.15. Molecular study

3.15.1. RNA Purification

RNA was isolated from the sample according to the protocol of TRIzol™ Reagent in the following steps:

A. Cell lysis

For each tube, 0.25 mL of blood was added to 0.75 mL of TRIzol™ Reagent, and the lysate was homogenized by pipetting several times.

B. Three phase separations

• Two hundred microliters of chloroform were added to the lysate, and then the tube cap was secured.

• The mixture was incubated for 2–3 minutes (at room temperature), and centrifuged for 10 minutes at 12,000 rpm. Subsequently, the mixture was separated into a lower organic phase, an interphase, and a colorless upper aqueous phase.

• The aqueous phase containing the RNA was transferred into a new fresh tube.

C. RNA precipitation

• five hundred microleter of isopropanol were added to the aqueous phase, incubated for 10 minutes, and then centrifuged for 10 minutes at 12,000 rpm.

• Total RNA was precipitated and formed a white gel-like pellet at the bottom of the tube.

• Supernatant was discarded.

D.RNA washing

• five hundred microliters of 70% ethanol were added and vortex briefly, then centrifuged for 5 minutes at 10000 rpm., ethanol was removed and the pellet dried at room temperature.

E. RNA solubility

Pellet was resuspended in 100 µl of Nuclease Free Water and then incubated in a heat block set at 55–60°C for 10–15 minutes.

3.15.2. Determination of RNA concentration

3.15.2.1. Fluorescence Method

Quantus Fluorometer was used to detect the concentration of extracted RNA in order to detect the quality of samples for downstream applications. For 1 µl RNA, 200 µl of diluted QuantiFlour Dye was added and mixed. RNA concentration was measured after five min. of incubation at room temperature in a dark condition.

3.15.3. Primer preparation

On delivery from the supplier (Macrogen Company) all primers were dissolving in the required volume of nuclease-free water following the company instructions to give a final concentration of 100 pmol/µl as a stock solution. The working stock for each primer was prepared at a 10 µM concentration (Table 3.9).

Table (3.9) primers preparation for RT-PCR protocol.

Primers	Vol. of nuclease-free water (µl)	Concentration (pmol/µl)
SOD1-F	300	100
SOD1-R	300	100
Mn-SOD –F	300	100
Mn-SOD-R	300	100
B-actin –F	300	100
B-actin-R	300	100

3.15.4. Reaction Setup and Thermal Cycling Protocol

3.15.4.1.One Step RT-PCR

PCR Component Calculation				
No. of Reaction	65	Rxn	Annealing temperature of primers pairs.	52,60,62
Reaction Volume /run	10	µl	No. of primers pairs	3
Safety Margin	5	%	No. of PCR Cycles	40

Mastermix components	Stock	Unit	Final	Unit	Volume for 1 Sample
qPCR Master Mix	2	X	1	X	5
RT Mix	50	X	1	X	0.25
MgCl2					0.25

Real Time PCR Program				
Steps	C°	m:s	Cycle	
RT. Enzyme Activation	37	15:00		1
Initial Denaturation	95	05:00		
Denaturation	95	00:20		40
Annealing	*52, 60 or 62	00:20		
Extension	72	00:20		

***SOD1 52ºC, MnSOD 60ºC, B-actin 62ºC**

3.16. Calculation of Gene Expression (Gene Fold)

To evaluate qPCR results, there are two approaches, Absolute and relative quantification. Absolute quantification uses a standard curve produced by Livak and Schmittgen to determine the quantity of input genes. (Livak & Schmittgen, 2001).On the other hand, Pfaffl's relative quantification evaluates changes in gene expression compared to a reference genes sample. (Pfaffl, 2001).

Gene expression or gene fold or RQ (Relative quantification) value calculated by Pfaffl equation (Pfaffl, 2001):

$$RQ = 2^{-(\Delta\Delta CT)}$$

Gene fold or RQ is calculated firstly by collecting CT (CT -threshold cycle or CQ-quantification cycle) average value from real-time PCR device for each sample then calculating ΔCT value for samples as follows:

Δ CT = CT (gene of interest) – CT (reference gene)

ΔCT is the difference in CT values for the gene of interest and the reference gene for a given sample. This will normalize the gene of interest to a gene not affected by the experiment (housekeeping gene)

Then Calculating ΔΔCT value is as follows:

ΔΔ CT = Δ CT (treated sample) – Δ CT (untreated sample (control))

After calculating ΔΔCT for all samples then take the final equation to calculate gene expression or RQ as follow:

Fold gene expression RQ = $^{2-}$(ΔΔCT)

3.17. Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism7 software. One-way ANOVA and t-test were used to find P-value. The Pearson correlation coefficient was used to investigate the correlation between the studied parameters. A difference of $P<0.05$ and $P<0.01$ was considered statistically significant. All data were presented by using means and standard deviation (stdv.)

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3. Materials and Methods

3.1. Materials

3.1.1 . Equipment

Table (3.1) Equipment and apparatus used in this study

Device	Company	Origin
Cooling centrifuge	Hittch	Germany
Laminar flow UV cabinet	Labtech	Korea
Vortex Mixer	Witeg	Germany
Centrifuge	Fisher Scientific	USA
Mic qPCR Cycler	Bio Molecular System	Australia
Mic Tube	Bio Molecular System	Australia
Quantus Fluorometer	Promega	USA

3.1.2. Chemicals

Table (3.2): Chemical materials used in this study.

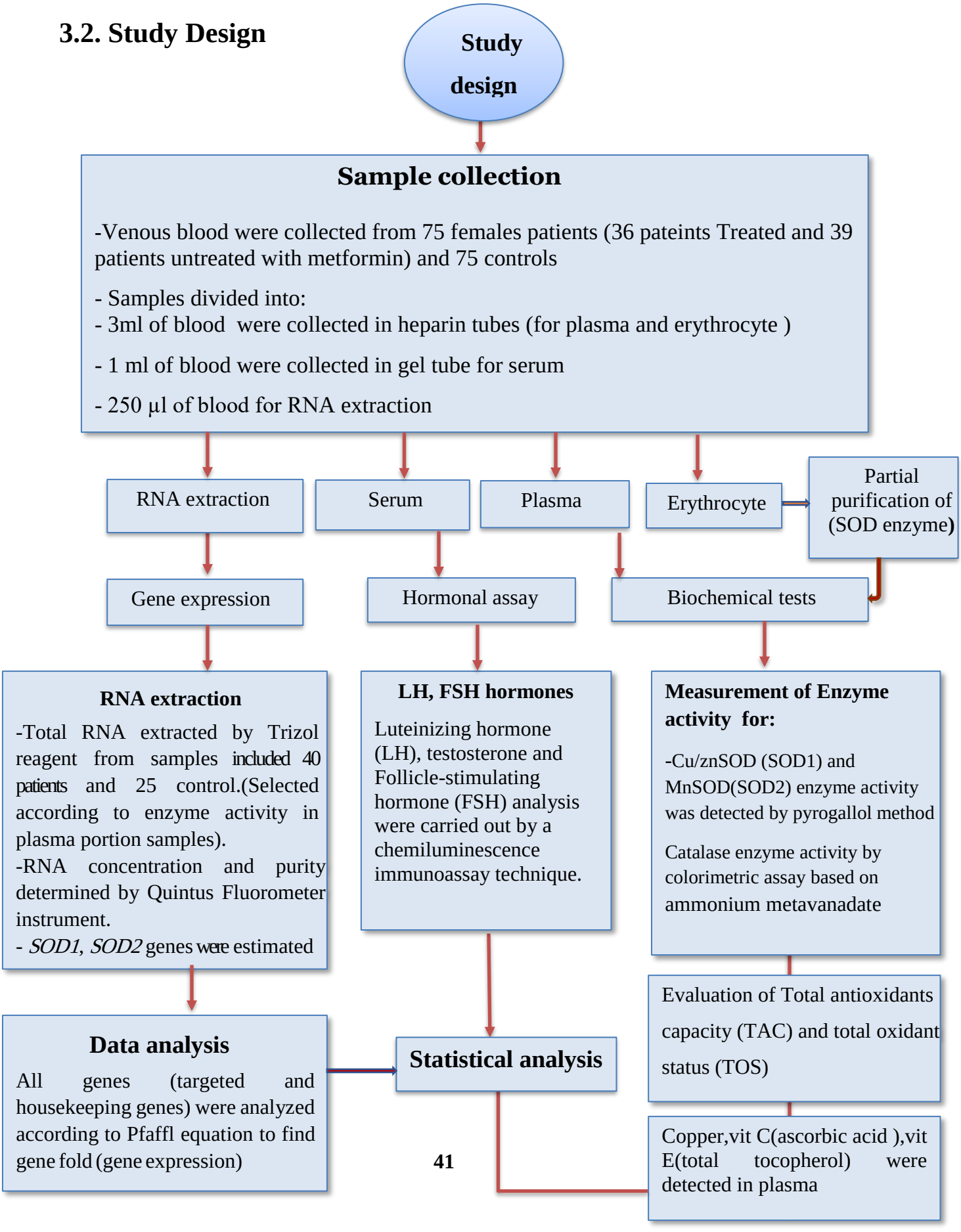
Chemical materials	Manufacture	Origin
Isopropanol	High Media	India
TRIzol Reagent	Thermo fisher Scientific	USA
Chloroform	LiChrosolv	Germany
Primers	Macrogen	Korea
GoTaq® 1-Step RT-qPCR System, MgCL2, Nuclease Free Water, Quantifluor RNA System	Promega	USA

3.1.3. Primers

Table (3.3): Primers of *SOD1*, *SOD2* gene and *β-actin*(housekeeping gene) used in this study

Primer name	Sequence 5` ⟶ 3`	Annealing Temp.(C)	References
***SOD1*-F**	**CGAGCAGAAGGAAAGTAATG**	**52**	**(Sugino *et al.*, 2000)**
***SOD1*-R**	**TAGCAGGATAACAGATGAGT**		
***SOD2*-F**	**AGTTCAATGGTGGTGGTCATA**	**60**	
***SOD2*-R**	**CAATCCCCAGCAGTGGAATAA**		
***β-actin*-F**	**CCCTGGACTTCGAGCAAGAG**	**62**	**(Rong *et al.*, 2016)**
***β-actin*-R**	**TCACACTTCATGATGGAGTTG**		

3.2. Study Design



3.3. Subjects

A total number of 75 females with PCOS were involved in this study. samples were collected during the period from November 2019 until October 2021, the samples were collected from females (married and unmarried/ patients and controls) who regularly visited Imam AL-Sadeq hospital and private gynaecology clinic in AL- Hillah city whose ages ranged from (20-45) years, Metformin treatment were included in this study in which PCOS women divided into two groups: PCOS patients treated with metformin (36 patients)and PCOS patients untreated with metformin (39 patients). (Participants were provided with an appropriate understanding of the research work and written informed agreement was provided by participants before collecting samples or any data. Related information about the participants', socio-demographic and health history were collected by using questionnaires.). Physicians evaluated the clinical assessment of patients with PCOS according to sonograph and laboratory assessment; hormone levels FSH, LH were estimated. The questionnaire form has been filled out for each patient and control as shown in appendix (A).

3.3.1. The inclusion criteria used for the recruitment of PCOS subjects

PCOS was diagnosed according to Rotterdam criteria (Fauser *et al.*, 2012). Two out of three of the following criteria were met for the diagnosis:

1. Clinical and biochemical signs of hyperandrogenism.

Clinical: Hirsutism was considered the indicator of hyperandrogenism. Modified Ferriman-Gallwey (MFG) score test was taken as a sign of locations in the body, The MFG score evaluates hair growth in nine androgen-sensitive body areas: the upper lip, chin, chest, upper and lower back, upper

and lower abdomen, upper arm, and thigh. Hair distribution score ≥8 of 36 (Ilagan *et al.*, 2019) (Figure 3.1). **Biochemical:** Androgen values greater than the reference range (total testosterone and dehydroepiandrosterone sulfate (DHEA-S)) were determined as hyperandrogenemia.

2. Oligo ovulation or non-ovulation; Cycle ranged from 35-45 days (oligomenorrhoea) or absence of menstruation for more than 3 months (amenorrhoea).

3. Appearance of polycystic ovaries on ultrasound. In 3rd or 4th day of the menstrual cycle, ultrasonic evaluation was performed with transvaginal ultrasound to check the morphological appearance of the ovaries.

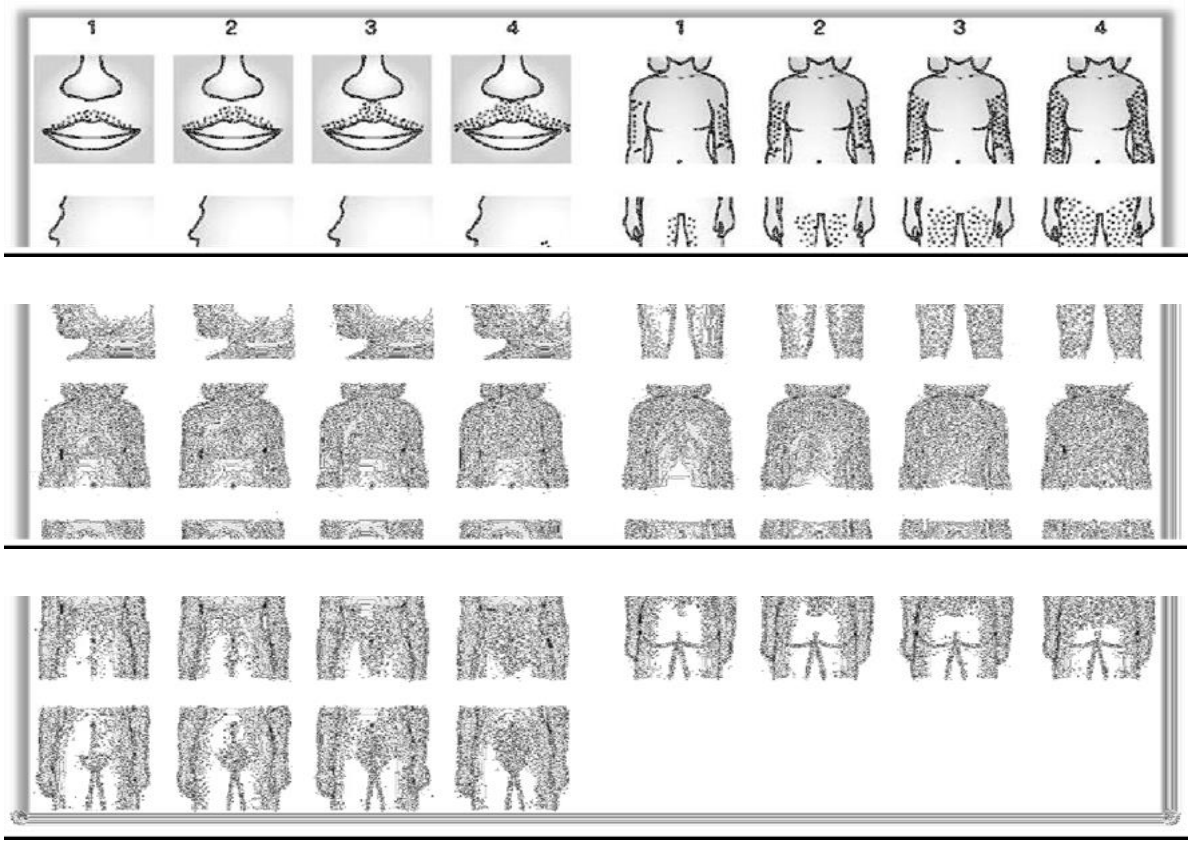


Figure (3.1) : Ferrimane Gallwey hirsutism scoring system (Hatch et *al.*, 1981).

3.3.2. The inclusion criteria used for the recruitment of the control group

1. The length of their menstrual cycles ranged between 22-35days.
2. History of spontaneous conception.
3. They had no clinical or biochemical signs of hyperandrogenism and no polycystic ovaries at any stage of life.
4. Body mass index (BMI) for both patients and the healthy control group was measured as follows: weight (kilogram), Height (m2).

3.3.3. Disorders that possibly mimic PCOS and are excluded from the Rotterdam criteria:

1. Hyperprolactinemia is an increase in prolactin levels above the reference range. Although elevated prolactin levels have been reported in women with PCOS, hyperprolactinemia and PCOS should be treated as two separate clinical entities (Davoudi *et al.*, 2021).

2. Thyroid disease, as measured by levels of thyroid-stimulating hormone (TSH), subjects with elevated or low TSH were excluded from this study.

3. Non-classical congenital adrenal hyperplasia, was excluded from the study.

4. women taking contraceptives or induction, **smoked women, or having hypertension or diabetes,** are all excluded.

3.4. Body Mass Index (BMI)

Body mass index (BMI) is commonly used as a substitute marker and indicates weight-for-height without considering differences in body composition and the contribution of body fat to overall body weight (Pasco *et al*., 2014) BMI (kg/m^2) = weight (kg) / $(height)^2$ $(m)^2$

There are four BMI categories:

1. BMI of fewer than 18.5 is considered underweight.

2. BMI values between 18.5 and 24.9 are considered a normal or healthy weight.

3. BMI values between 25 and 29.9 are considered overweight.

4. BMI 30 and above are considered obese (BMI 30-39.9 severely obese, BMI 40-49.9 morbidly obese, and BMI>50 represents super obese).

3.5. Collection of Blood and Sample Preparation

Five ml of blood was obtained from each participant by vein puncture, The blood sample was obtained during the early follicular phase of a menstrual cycle (days 2–5). Blood samples were collected in heparin tubes (for plasma and erythrocyte, approximately 2 ml of blood), 2 ml of blood in a gel tube for serum separation, and 250 microliters of blood with 750 microliters of trizol for RNA extraction.

3.5.1. Preparation of erythrocyte

Blood samples were centrifuged at 1000 × g for 10 min at 4°C to remove the plasma. The buffy coat on the erythrocyte sediment was separated carefully after the plasma was removed. The plasma portion was frozen at -20°C until the time of analysis. Erythrocytes (packed cells) were washed 3 times by centrifugation with 3 volumes of cold 0.15 M NaCl (0.9-gram NaCl in 100 ml DW.) at 4000 g for 5 min. The cells were frozen at -20 °C and stored or lysed by thawing. The hemolysate was diluted by adding 3 volumes of ice-cold deionized water. The temperature was maintained at 0-2 °C by using an ice water mixture while ethanol was added with adequate stirring to a final concentration of 25% (v/v). Cold chloroform was added to the mixture to a concentration of 12% (v/v). Stirring was continued for 30 minutes, during

which the haemoglobin was rendered insoluble, and centrifuged for 10 minutes at 3000 rpm. The enzyme is contained in the clear top layer. The resulting supernatant was allowed to warm at room temperature, and solid K_2HPO_4 (300g/l) was added, separating two liquid phases. The denser phase was essentially aqueous and contained most of the salts, the lighter phase was ethanol and contained SOD and little salts. The upper phase was collected and centrifuged. The supernatant contained all of the SOD activity(Djalali *et al.*, 2005).

3.6. Superoxide Dismutase (SOD) enzyme activity

3.6.1. Principle

(Cu\Zn) SOD enzyme activity was determined by used a simple and rapid method based on the ability of the enzyme to inhibit the autoxidation of pyrogallol. The autoxidation of pyrogallol in the presence of EDTA in pH (8.2) is 50%. The Principle of this method is based on the competition between pyrogallol autoxidation by $O_2\bullet^-$ and the dismutation of this radical by SOD(Marklund & Marklund, 1974).

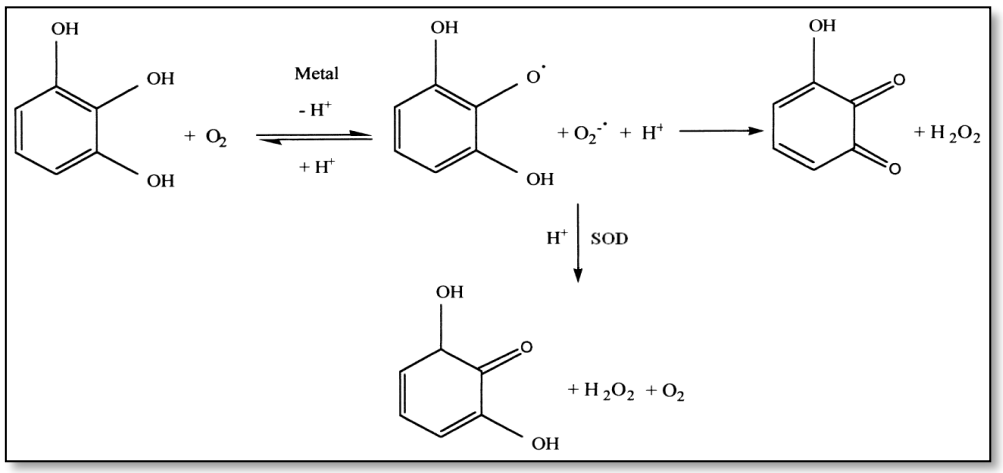


Figure (3.2): Suggested Mechanism of Pyrogallol Autoxidation.

(Cu\Zn) SOD activity is expressed as units/ml. One unit of (Cu/Zn) SOD activity is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation.

3.6.2.Reagents Preparation

1. Tris- EDTA buffer pH 8.2 A weight of 2.85 g of Tris and 1.11 g of EDTA-Na_2 were dissolved in 1 liter of DW. Adjusted with 0.1 M HCl into pH 8.2.

2. Pyrogallol Solution (0.2 mM) A weight of 0.252 g of pyrogallol was dissolved in a solution of 0.6 ml of concentrated hydrochloric acid diluted in 1 liter of DW

3.6.3. Procedure

A spectrophotometer was adjusted to read zero using a Tris-EDTA buffer. Control and sample test tubes were prepared and then pipetted into test tubes

Reagents	Test (µl)	Control (µl)
Sample	50	-
Tris-buffer	1000	1000
DW	-	50
Pyrogallol	1000	1000

Absorption read at the wavelength of 420 nm against Tris-EDTA buffer at zero time and after 1 minute and after 2 minutes of the pyrogallol addition.

3.6.4. Calculation of SOD activity:

$$\% \text{ Inhibition of pyrogallol autoxidation} = \frac{\Delta A \text{ CONTROL} - \Delta A \text{ test}}{\Delta A \text{ control}} \times 100\%$$

$$\text{SOD Activity (U/ml)} = \frac{\text{inhibition of pyrogallol autoxidation}}{50\%} \times \%$$

*ΔA = Absorption after 2 min – Absorption at zero time

3.7. Manganese Superoxide Dismutase (MnSOD) enzyme activity

3.7.1. Principle

MnSOD enzyme activity was determined according to the method described by (Marklund and Marklund,1974; and Del Maestro *et al.*,1983), which is based on the ability of MnSOD to inhibit the auto-oxidation of pyrogallol in the presence of sodium cyanide to determine SOD activity in the sample.

3.7.2. The reaction mixture:

The reaction mixture consisted of

1. Tris- EDTA buffer pH 8.2
2. Pyrogallol Solution (0.2 mM)
3. 0.1 M NaCN (1 liter of Assay buffer containing 4.901gm NaCN)
4. Sample.

Cu\Zn SOD activity was inhibited by adding (1000 µl) of assay buffer containing NaCN for 15 min; the reaction was initiated by adding (1000 µl) of pyrogallol (final concentration of 0.2 mM), and the absorbance was measured at 420 nm. One unit of activity is defined as the amount of sample needed to inhibit pyrogallol oxidation by 50 %. The final results were expressed as U/ml(Stojkovski *et al.*, 2013).

3.7.3. Calculation of SOD activity:

$$\% \text{ Inhibition of pyrogallol autoxidation} = \frac{\Delta A \text{ CONTROL} - \Delta A \text{ test}}{\Delta A \text{ control}} \times 100\%$$

$$\text{SOD Activity (U/ml)} = \frac{\text{inhibition of pyrogallol autoxidation}}{50\%} \times \%$$

*ΔA = Absorption after 2 min – Absorption at zero time

3.8. Catalase enzyme activity

3.8.1. Principle

Catalase enzyme activity in biological fluids and tissues was determined by a colourimetric assay in which H_2O_2 dissociation rates are directly proportional to catalase activity. The Principle of this procedure was based on reactions of ammonium metavanadate with H_2O_2 under acidic conditions. The resulting reduction of vanadium (V) to vanadium (III) by H_2O_2 produces a red-orange peroxovanadium complex. The reaction between vanadium and H_2O_2 is shown in the following equation:

$$H_2O_2 + NH_4VO_3 + H_2SO_4 \rightarrow NH_4[VO(O_2)SO_4] + 2H_2O$$

Catalase enzyme activity was determined by monitoring the absorption of the red-orange peroxovanadium complex at 452 nm (Hadwan & kadhum Ali, 2018).

3.8.2. Reagents preparation

1. Sulfuric acid solution (0.5 M) was prepared by appropriate dilution of concentrated sulfuric acid in 200 ml of distilled water.

2. Ammonium metavanadate solution (0.01 M) contained 0.2925 g of ammonium metavanadate in 200 ml of 0.5M sulfuric acid.

3. Phosphate buffer (50 mM; pH 7.0) was prepared by mixing solutions (a) and (b) at a ratio of 1:1.5 a:b. **solution (a)** was prepared by dissolving 6.81 g of KH_2PO_4 in one liter of distilled water, and **solution (b)** was prepared by dissolving 8.90 g of $Na_2HPO_4.2H_2O$ in one liter of distilled water.

4. **Fresh H_2O_2 (10 mM)** solutions were prepared by mixing 0.1134 ml of 30% H_2O_2 with 100 ml of phosphate buffer, and the solution was adjusted to 10mM by using the molar extinction coefficient of H_2O_2 at 240 nm (43.6 $M^{-1}cm^{-1}$).

3.8.3. Procedure

Following (table 3.4) is the procedure for catalase activity analysis.

Table (3.4): Procedure for assessments of catalase activity.

Reagents	Test	Standard	Blank
Sample	100 µl	------	-----
Phosphate buffer	900 µl	1000 µl	3000 µl
Hydrogen peroxide	2000 µl	2000 µl	----
Mix with vortex and incubate at 37 °C for 2 min; after that, add:			
Vanadium reagent	2000 µl	2000 µl	2000 µl
After that, the tubes were kept at 25 °C for 10 min. The changes in absorbance were recorded at 452 nm against the blank.			

3.8.4. Calculation

The enzyme activity procedure was elucidated in (Table 1). The rate constant (k) of the first-order reaction equation for catalase activity was calculated using the following formula:

$$\text{Catalase Activity of test kU} = \frac{2.303}{t} * \frac{\log S^o}{S}$$

where t is time, S^o is the absorbance of the standard solution, and, S is the absorbance of the sample, and kU (katal unite)

3.9 Determination of Total Oxidant Status (TOS).

3.9.1. Principle

TOS of the sample was measured by using a method developed by Erel O 2005. Oxidants in the plasma oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is improved by glycerol molecules abundantly found in the reaction medium. The ferric ion creates a colored complex with xylenol orange in an acidic medium. Color intensity, which can

be determined spectrophotometrically, is associated with the sample's total amount of oxidant molecules. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 Eq/L) (Erel, 2005).

3.9.2. Reagents

Reagent 1: was prepared by dissolving 1.96 g of ferrous ammonium sulfate and 3.17 g of O-dianisidine dihydrochloride in 1000 mL of 25 mM H_2SO_4 solution. The final reagent consisted of 5 mM ferrous ammonium sulfate and 10 mM O-dianisidine dihydrochloride.

Reagent 2: was prepared by dissolving 114 mg of xylenol orange and 8.18 g of NaCl in 900 mL of 25 mM H_2SO_4 solution. One hundred milliliters of glycerol was added to the solution. The final reagent was composed of 150 µM xylenol orange, 140 mM NaCl and 1.35 M glycerol. The pH value of the reagent was 1.7. This reagent is stable for at least 6 months at 4°C.

Hydrogen Peroxide (STD): (100 µmol/L) was freshly diluted and standardized daily by using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm .

3.9.3. Procedure: Following (table 3.5) is the procedure for TOS analysis.

Table (3.5): Shows the Details of the TOS analysis method.

	Blank	Standard	Sample
Distilled water	100 µl	-----	------
Sample	------	-----	100 µl
Hydrogen peroxide	------	100 µl	------
R1	1 ml	1 ml	1 ml
Test tubes were mixed by vortex, then added:			
R2	0.25 ml	0.25 ml	0.25 ml
Gently mix the content of every tube after addition, let to stand at room temperature for 30 minutes, read spectrophotometrically at 560 nm.			

3.9.4. Calculation

$$\text{Total oxidants status} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD$$

3.10.Total Antioxidants Capacity Assay (TAC): The CUPRAC method.

3.10.1. Principle

The CUPRAC assay estimates the capacity of the antioxidants in samples to reduce the Cu^{2+} to Cu^{1+} in the presence of a chelating agent. These chelators form a stable color complex with Cu^{1+} that have absorption at 450 nm. The CUPRAC assay measures thiol-group antioxidants and other plasma antioxidants such as ascorbic acid, α-tocopherol, β-carotene, uric acid, albumin, and bilirubin.

Antioxidants + Cu^{+2} ⟶ Cu^{+1}

Cu^{+1} + 2, 9-dimethyl-1, 10-phenanthroline ⟶ complex (λ_{max} at 450 nm)

(Apak *et al.*, 2008).

3.10.2.Reagents

1. Copper(II) chloride solution at a concentration of 10^{-2}M was prepared by dissolving 0.4262 g of $CuCl_2 .2\ H_2O$ in 250 ml of distilled H_2O.

2. Ammonium acetate (NH_4Ac) buffer pH = 7.0 was prepared by dissolving 19.27 g of NH_4Ac in water and completing the volume up to 250 ml.

3. Neocuproine (Nc) solution at a concentration of $7.5x\ 10^{-3}$M was prepared by dissolving 0.039 g Nc in 96% EtOH, and the volume was completed to 25 ml with ethanol.

4. The standard solutions of sample antioxidants were prepared at 1.0×10^{-3} concentration of Trolox.

3.10.3.Procedure

Following (table 3.6) is the procedure TAC analysis by the **CUPRRAC method**.

Table (3.6) Shows the Details of the CUPRRAC method.

Reagents	Test	Standard	Blank
Copper(II) chloride solution	1ml	1ml	1ml
Sample	50 µl	------	-------
Working standard solution	-------	50 µl	-------
D.W	-------	-------	50 µl
Neocuproine (Nc) solution	1ml	1ml	1ml
Ammonium acetate (NH_4Ac) buffer	1ml	1ml	1ml

3.10.4.Calculation

$$\text{Total antioxidants levels} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD\left(\mu mol / l\right)$$

3.11. Measurement of free copper by (LTA Kit)

3.11.1. Principle

In the copper test, chromogen 3,5-Di-Br-PAESA was reacted with cupric ions and formed a blue-violet compound; the color intensity is proportional to the copper concentration in the sample

3.11.2. Reagents

Reagent A: Acetate buffer 0.1M, PH 4.9 (prevents the pH of a solution from changing drastically)

Reagent B : 3,5-Di-Br-PAESA

Standard: Ion copper 200 µg/dl

3.11.3. Procedure

A. Determination of copper in plasma: Reagent preparation includes preparing of working reagent by mixing an equal quantity of Reagent A with Reagent B, which remains stable for 20 days at room temperature.

Table (3.7): procedure for copper determination in plasma.

Reagents	Test	Standard	Blank
Work reagents	1ml	1ml	1ml
Distilled water	66µl	…..	……..
Standard	……..	66µl	…….
Plasma	……..	……	66µl

Mix and wait for 10 min, then read the absorbance against the blank at 580 nm.

B. Calculations

Copper µg/dl = A(Sample)\ A (Standard) * 200

3.11.4. Normal values

Man	**80- 140 µg/dl**
Woman	**80-155 µg /dl**

3.12. Assay of total tocopherol (vitamin E) in plasma

3.12.1. Principle

Plasma total tocopherol was assayed by the method of (Quaife *et al.*, 1949), It involves the Emmerie- Engel color reaction with ferric chloride and α,α-dipyridyl to give a red color

3.12.2. Reagents:

1. absolute ethanol.

2.α,α-dipyridyl: was prepared by dissolving 0.120 gm of α,α-dipyridyl in 100 ml of n-propyl alcohol.

3. Ferric chloride hexahydrate: was prepared by dissolving 0.120 gm of Ferric chloride hexahydrate in 100 ml of absolute ethanol. This solution was kept in a dark brown or red glass bottle.

4.α-tocopherol standard (1µmol/L) was prepared by dissolving 2.0 mg of α-tocopherol in 100 ml of absolute ethanol.

3.12.3. Procedure:

Following the procedure of vit E determination in plasma.

Table (3.8): procedure of vit E determination in plasma.

Reagents	Test	Standard	Blank
Absolute ethanol	0.6 ml	0.6 ml	0.6 ml
Sample	0.6 ml	------	-------
D.W.	-----	------	0.6 ml
STD	-----	0.6 ml	-------

Xylene	0.6 ml	0.6 ml	0.6 ml
Mixed well and centrifuged for 10 min at 3000 rpm.			
xylene supernatant layer	0.4 ml	0.4 ml	0.4 ml
α,α'-dipyridyl was added and vortexed	0.4 ml	0.4 ml	0.4 ml
The 0.6 ml of this mixture was pipetted into a cuvette and the absorption was measured spectrophotometrically at 460 nm against deionized water.			
ferric chloride	0.13 ml	0.13 ml	0.13 ml
Mixed thoroughly and absorption was again read at 520 nm spectrophotometrically exactly 1.5 min after the addition of ferric chloride.			

3.12.4. Calculation

$$\text{Conc. of test} = \frac{(\text{A}\,520 - 0.29\,\text{A}\,460)\text{test}}{\text{A}\,520\text{STD}} * \text{Conc. of STD}$$

3.13. Determination of the Total Vitamin C (Ascorbic Acid) in plasma

3.13.1. Principle

In the 2,4-dinitrophenylhydrazine (DNPH) methods, Ascorbic acide is oxidized by Cu+2 to DHA (dehydroascorbic acid) and diketogulonic acid (Burtis, 1999). When treated with DNPH (Dinitrophenyl hydrazine), the 2,4-dehydrophenylosazon product forms, which, in the presence of sulfuric acid, forms an orange-red complex that absorbs at 520 nm as shown in (figure 3.3

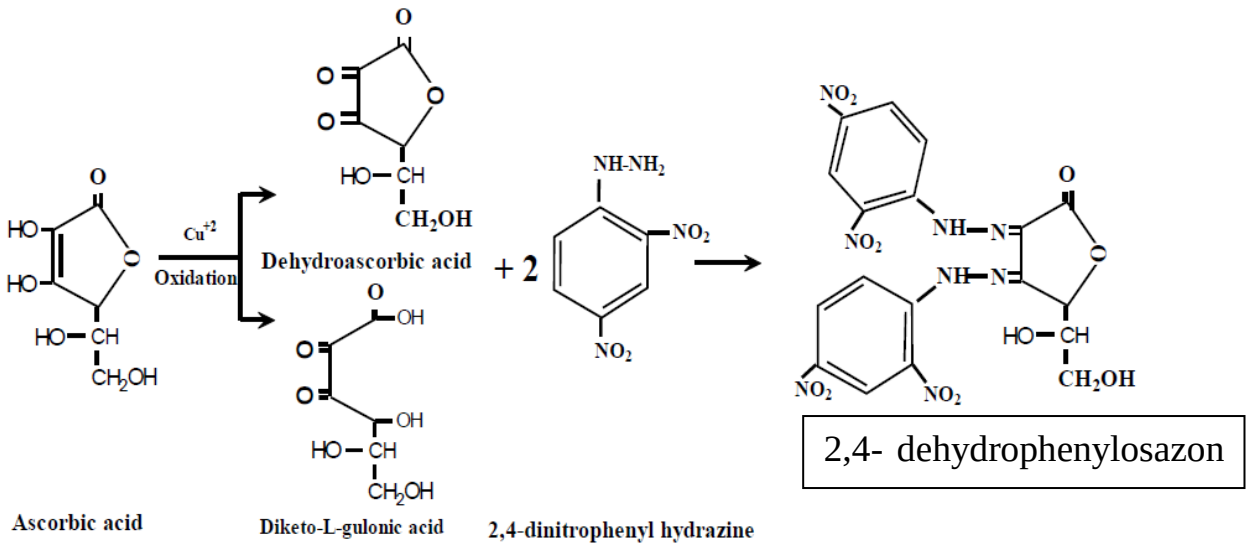


Figure (3.3): Reaction Between Ascorbic Acid and 2,4-DNPH (Lloyd *et al.,* 1945).

3.13.2. Preparation of Reagents

1. Metaphosphoric acid (m-HPO3) (0.75M) 30gm of m-HPO3 are dissolved in a final volume of 500 ml ddH_2O. (Stable for 1 week).

2. Sulfuric acid H2SO4 (4.5M) Carefully 250 ml of concentrated H2SO4 are added to 500 ml of cold ddH_2O. When the solution has been cooled at room temperature, ddH_2O is added up to 1 liter, with mixing (Stable for 2 years).

3.Sulfuric acid H2SO4 (12M) Carefully 650 ml of concentrated H_2SO_4 are added to 300 ml of cold ddH_2O and brings to a final volume of 1 liter (Stable for 2 years).

4. 2,4-DNPH (2,4-Dinitrophenyl hydrazine) reagent (0.01M) 10 gm of 2,4-DNPH were dissolved in 4.5 M H_2SO_4 and bring to a final volume of 500

ml, then refrigerated overnight, and filtered. (Stable for at least 1 week at refrigerated temperature).

5. Thiourea (0.66M) 5 gm of thiourea are dissolved in a final volume of 100 ml of ddH_2O. (Stable for 1 month at 4C°).

6. Copper sulfate (0.027M) 0.6 gm of anhydrous copper sulfate is dissolved in a final volume of 100 ml of ddH_2O. (Stable for 1 year at room temperature).

7. DTCS reagent: 100 ml of the 2,4-DNPH reagent, 5 ml of the thiourea,and 5 ml of the copper sulfate are combined. (Store in bottle at 4C° for a maximum of 1 week).

8. Ascorbic acid standards Stock standard solution (2.8 mM) is prepared by dissolving 50 mg of ascorbic acid in a final volume of 100 ml of m-HPO_3. Dilutions are made by m-HPO_3 to 2.5, 5 and 20 mg/L (0.014, 0.028, and 0.11 mM) respectively. There are the working standards (All working standards should be prepared freshly).

3.13.3. Procedure

The procedure for determination of total vitamin C in plasma by 2,4-DNPH method is summarized as follows: Each standard and sample test tube are prepared, then pipetted into test tubes

Reagents	Sample(µL)
m-HPO3 **plasma**	800 200

Tubes are mixed in vortex mixture, then centrifuged at 2500 x g for 10 minutes

Reagents	Sample(µL)	Reagent Blank(µL)	Standard(µL)
Supernatant	600		
Standards			600
m-HPO3		600	
DTCS reagent	200	200	200

Tubes are capped and mixed in a vortex mixture, then incubated in a water bath at 37C° for 3 hours.

The tubes are removed from the water bath and chilled for 10 minutes in an ice bath, with slowly mixed

Reagents	Sample(µL)	Reagent Blank(µL)	Standard(µL)
Cold H2SO4 (12M)	1000	1000	1000

Tubes are mixed in a vortex mixture and returned immediately to the ice bath. The spectrophotometer is adjusted with blank to read zero absorbance (A) at 520 nm, and the absorbance of standards and sample is read.

3.13.4. Calculation of plasma Vitamin C

The concentrations of the samples were obtained from the calibration curve (Figure 3.4) and were multiplied by 5 to normalize the dilution of the plasma m- HPO3) to give the concentration of vitamin C (ascorbic acid) per liter of sample. The concentration of ascorbic acid can be determined directly from a standard as follows:

$$\frac{\text{A sample}}{\text{C sample}} = \frac{\text{Astd}}{\text{C std}}$$

$$\text{C sample} = \frac{\text{C std}}{\text{A std}} * \text{A sample} * 5$$

where: Astd and Cstd

C = concentration mg/L of AA in sample and standard.(std)

A = absorbance at 520 nm for sample and standard.

5 = factor is added to normalize the dilution of samples.

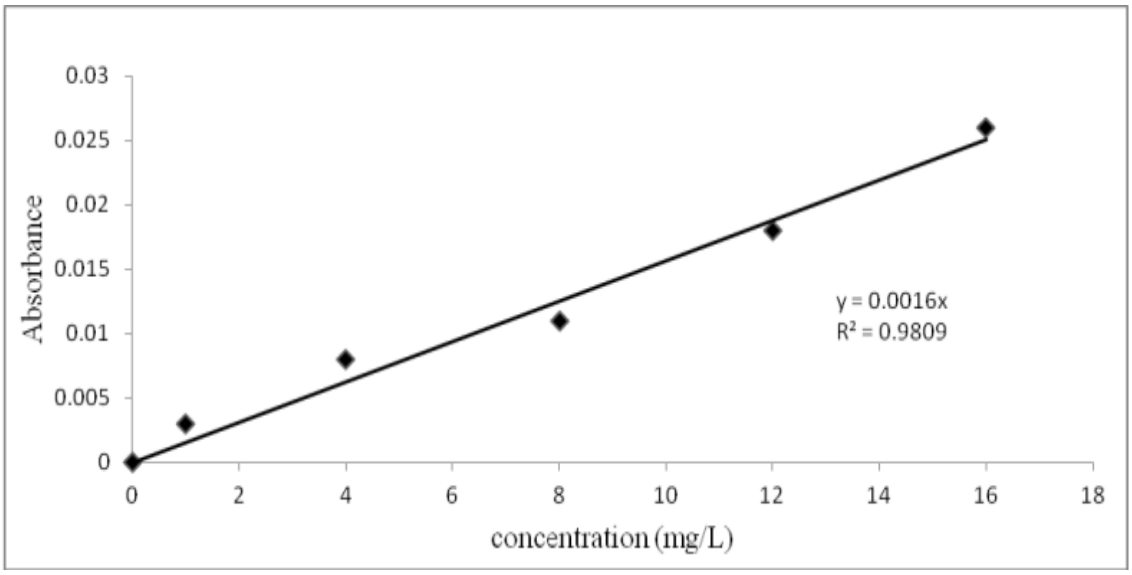


Figure (3.4): Standard curve of ascorbic acid

3.14. Determination of serum Hormones

3.14.1. Determination of Luteinizing Hormone (LH) by using MAGLUMI LH (CLIA) Kit

3.14.1.1 Principle

Method is used an anti-LH monoclonal antibody to label *N*-(4-aminobutyl)-*N*-ethyl-isoluminol (ABEI), and used another monoclonal antibody to coat magnetic microbeads. Sample, Calibrator or Control, with ABEI Label and magnetic microbeads are mixed thoroughly and incubated at 37℃, forming a sandwich; After sediment in a magnetic field, suck the supernatant and then cycle wash it for one time. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as as Relative Light Units (RLU) within 3 seconds and is proportional to the concentration of LH present in samples

3.14.1.2. Reagents integral preparation

1. Microbeads were mixed prior to loading the reagent assembly onto the system for the first time to resuspend the microbeads that had settled during shipment.
2. The microwheel in the microbead chamber was rotated back and forth until the color of the suspension changed to brown, waited for 30 minutes for the integrator to sit in the reagent compartment. The magnetic microbeads are automatically agitated and completely suspend during this period
3. The solutions were stored at 2-8 °C before use and are good until the expiration date. The solutions are stable for 30 days after they are

opened. When not in use, open solutions should be stored in the refrigerator for the greatest results

3.14.1.3. Procedure

1. The device reagents have been downloaded after scanning the device space automatically in the device test, the group has been suspended on the bead in the automatic confusion solution that occurs after loading for 30 minutes and then the detector and sample as well as to increase stability.

2. The volume of 40μL sample has been added, the control tube, the intense location, and the device has been entered, and the beginning was specified.

3. The sample was mixed with maglumi system ABEI label + 80μl, nanomagnetic microbeads + 20μl then incubated for 15-minute at 37 ° C.

4. Cuvette was transferred to washing station to a cycle of washing for 3 times to remove the illegal content by 400 μl washing buffer (one bottle of concentrated for concentrated washing to 10 liters for this purpose.

5. Cuvette has been moved to a triangular measurement captured by the room that is a dark room, here, 2 substrates. Starter 1+ 2 Then is run with the Current Chemiluminescence label: ABEI, the light signal is measured by a photomultiplier within 3 seconds

6. After finished measured, Cuvette has been paid to the waste bag and the end of the test.

3.14.1.4. Calculations

The starter detector has been added to start the chemiluminescent interaction, and the production of a light signal is measured by photomultiplier, which suit the LH concentration in the sample, the analyzer automatically calculates LH concentration in each sample through the calibration curve created by the calibration curve 3 points. The results are expressed in mIU / ml.

3.14.2. Determination of serum Follicle-stimulating hormone (FSH) concentration by using MAGLUMI LH (CLIA) Kit

3.14.2.1. Principle

Sandwich immunoluminometric assay was used an anti-FSH monoclonal antibody to N-(4-aminobutyl)-N-ethyl-isoluminol (ABEI) label, and has been used another monoclonal antibody to coat magnetic microbeads. Sample, Calibrator or Control, with ABEI Label and magnetic microbeads are mixed thoroughly and incubated at 37℃, forming a sandwich; After sediment in a magnetic field, suck the supernatant and then cycle wash it for 1 time. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as Relative Light Units (RLU) within 3 seconds and is proportional to the concentration of FSH present in samples

3.14.2.3. Procedure

1. The reagents were loaded into the device after scanning the code using the automatic area scanning of the device in order to take the test device, the group containing the bead was suspended in the solution via the automatic mixing that occurs after loading for 30 minutes, and then the reagent and sample area was cooled to increase stability.

2. A volume of 40µL of sample and control was placed in a collection tube, entered the site and condenser device, and then the starting was determined.

3. Sample needle and reagent sampler were taken into a cuvette; serum was combined with AbEI Label + 80µL and Nanomagnetic Microbeads + 20 µL from the Maglumi System kit.

4. Mixture was incubated at 37 °C for 15 minutes.

5. The cuvette was transferred to the washing station and washed cycle washed three times with 400 L wash buffer to remove non-agglutinating contents (concentrated wash contents are diluted to 10 L for this purpose).

6. The cuvette was moved to a three-second fitting room, which is a dark room, and two braces were used, starters 1 and 2 were applied, and they reacted with the mark of ABEI Chemiluminescence.

7. The cuvette was pushed into the garbage bag after being measured, and the test was completed.

3.14.2.4. Calculation Result

The initiator reagent was added to start a luminous chemical reaction, which resulted in an optical signal measured in a photomultiplier as relative luminous units (RLUs) which are proportional to the concentration of FSH present in the sample, and the analyzer automatically calculated the FSH concentration in each sample by means of the generated calibration curve By performing a two-point master calibration curve. The results were expressed in mIU/ml,The sample concentrations were between 0.02-78.92mIU/ml.

3.15. Molecular study

3.15.1. RNA Purification

RNA was isolated from the sample according to the protocol of TRIzol™ Reagent in the following steps:

A. Cell lysis

For each tube, 0.25 mL of blood was added to 0.75 mL of TRIzol™ Reagent, and the lysate was homogenized by pipetting several times.

B. Three phase separations

• Two hundred microliters of chloroform were added to the lysate, and then the tube cap was secured.

• The mixture was incubated for 2–3 minutes (at room temperature), and centrifuged for 10 minutes at 12,000 rpm. Subsequently, the mixture was separated into a lower organic phase, an interphase, and a colorless upper aqueous phase.

• The aqueous phase containing the RNA was transferred into a new fresh tube.

C. RNA precipitation

• five hundred microleter of isopropanol were added to the aqueous phase, incubated for 10 minutes, and then centrifuged for 10 minutes at 12,000 rpm.

• Total RNA was precipitated and formed a white gel-like pellet at the bottom of the tube.

• Supernatant was discarded.

D.RNA washing

• five hundred microliters of 70% ethanol were added and vortex briefly, then centrifuged for 5 minutes at 10000 rpm., ethanol was removed and the pellet dried at room temperature.

E. RNA solubility

Pellet was resuspended in 100 µl of Nuclease Free Water and then incubated in a heat block set at 55–60°C for 10–15 minutes.

3.15.2. Determination of RNA concentration

3.15.2.1. Fluorescence Method

Quantus Fluorometer was used to detect the concentration of extracted RNA in order to detect the quality of samples for downstream applications. For 1 µl RNA, 200 µl of diluted QuantiFlour Dye was added and mixed. RNA concentration was measured after five min. of incubation at room temperature in a dark condition.

3.15.3. Primer preparation

On delivery from the supplier (Macrogen Company) all primers were dissolving in the required volume of nuclease-free water following the company instructions to give a final concentration of 100 pmol/µl as a stock solution. The working stock for each primer was prepared at a 10 µM concentration (Table 3.9).

Table (3.9) primers preparation for RT-PCR protocol.

Primers	Vol. of nuclease-free water (µl)	Concentration (pmol/µl)
SOD1-F	300	100
SOD1-R	300	100
Mn-SOD –F	300	100
Mn-SOD-R	300	100
B-actin –F	300	100
B-actin-R	300	100

3.15.4. Reaction Setup and Thermal Cycling Protocol

3.15.4.1.One Step RT-PCR

PCR Component Calculation				
No. of Reaction	65	Rxn	Annealing temperature of primers pairs.	52,60,62
Reaction Volume /run	10	µl	No. of primers pairs	3
Safety Margin	5	%	No. of PCR Cycles	40

Mastermix components	Stock	Unit	Final	Unit	Volume for 1 Sample
qPCR Master Mix	2	X	1	X	5
RT Mix	50	X	1	X	0.25
MgCl2					0.25

Real Time PCR Program				
Steps	C°	m:s	Cycle	
RT. Enzyme Activation	37	15:00		1
Initial Denaturation	95	05:00		
Denaturation	95	00:20		40
Annealing	*52, 60 or 62	00:20		
Extension	72	00:20		

***SOD1 52ºC, MnSOD 60ºC, B-actin 62ºC**

3.16. Calculation of Gene Expression (Gene Fold)

To evaluate qPCR results, there are two approaches, Absolute and relative quantification. Absolute quantification uses a standard curve produced by Livak and Schmittgen to determine the quantity of input genes. (Livak & Schmittgen, 2001).On the other hand, Pfaffl's relative quantification evaluates changes in gene expression compared to a reference genes sample. (Pfaffl, 2001).

Gene expression or gene fold or RQ (Relative quantification) value calculated by Pfaffl equation (Pfaffl, 2001):

$$RQ = 2^{-(\Delta\Delta CT)}$$

Gene fold or RQ is calculated firstly by collecting CT (CT -threshold cycle or CQ-quantification cycle) average value from real-time PCR device for each sample then calculating ΔCT value for samples as follows:

Δ CT = CT (gene of interest) – CT (reference gene)

ΔCT is the difference in CT values for the gene of interest and the reference gene for a given sample. This will normalize the gene of interest to a gene not affected by the experiment (housekeeping gene)

Then Calculating ΔΔCT value is as follows:

ΔΔ CT = Δ CT (treated sample) – Δ CT (untreated sample (control))

After calculating ΔΔCT for all samples then take the final equation to calculate gene expression or RQ as follow:

Fold gene expression RQ = $^{2-}$(ΔΔCT)

3.17. Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism7 software. One-way ANOVA and t-test were used to find P-value. The Pearson correlation coefficient was used to investigate the correlation between the studied parameters. A difference of $P<0.05$ and $P<0.01$ was considered statistically significant. All data were presented by using means and standard deviation (stdv.)

We, the examiner committee, certify that we have read the thesis entitled " **Gene Expression of Superoxide Dismutase and Determination of Other Antioxidants associated with Polycystic Ovarian Syndrome "** and have examined the student " **Aghras Sabah Nawar Sallal"** in its content, and that in our opinion it is accepted as a thesis for degree of Doctorate of philosophy in Biology/Biotechnology with" **Excellent"** estimation.

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Abbreviation

Abbreviation	
AMP-K	Adenosine monophosphate activated protein kinase
cAMP	Cyclic AMP
Cat	Catalase
cDNA	Complementary Deoxy Nuclear Acid
CYPl7	cytochrome P450 family 17
DHA	Dehydroascorbic acide
FSH	Follicle Stimulating Hormone
GnRH	gonadotropin releasing hormone
IR	insulin resistance
LH	Luteinising Hormone
MDA	Malodialdehyde
MFG	Modified Ferriman-Gallwey
mRNA	Messenger RNA
m-HPO3	meta-Phosphoric acid
OS	oxidative stress
PCOS	Polycystic ovary syndrome
RIN	RNA Integrity Number
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
SHBG	Sex hormone binding globulin
SOD1	Cu/znSOD
SOD2	MnSOD
TAC	Total Antioxidants Capacity
TOS	Total Oxidant Status

Conclusions

1. Women with Polycystic ovarian syndrome exhibited increased Cu/znSOD, MnSOD enzyme activity with decreased catalase activity compared to controls. Suggesting that the byproduct of oxidative damage is expected to be raised in women with PCOS
2. The enzyme activity was more obvious in plasma fraction than in erythrocyte; thus, it can be considered as a biomarker for a polycystic ovarian syndrome
3. An increase in TOS level (in the plasma and erythrocyte samples) was found in women with PCOS, suggested that women with PCOS are under oxidative stress and supports the concept that oxidative stress is involved in PCOS pathophysiology.
4. A significant increase was found in the TAC level in the plasma and erythrocyte samples of PCOS patients than in normal women, in parallel with increased oxidative stress.
5. Increased zinc level in the polycystic ovary syndrome group with decreased ascorbic acid and tocopherol may be associated with oxidative stress within the body that puts the antioxidant defense system in a hyperactive state to compensate for this stress.
6. Treatment with Metformin drug led to reduc the oxidant status and total antioxidant capacity as this drug is responsible for reducing circulating insulin and increasing the glucose uptake by peripheral tissues.
7. SOD1 and catalase enzyme activity were significantly reduced in erythrocytes and plasma fractions due to reduced oxidative stress, while SOD2 showed a distinct pattern with enhanced enzyme activity in plasma and erythrocyte samples after metformin administration.

8. A significant association between PCOS and higher expression levels in *SOD1* and *SOD2* gene with increased activity was reprted. Treatment with Metformin drug was significantly related to a higher level of activity and expression of SOD2 , while lowering the expression of SOD1. These findings can open a new viewpoint in understanding the pathogenesis of PCOS, suggesting that OS might be involved in developing this syndrome.
9. There was a relationship between LH and FSH levels and antioxidant enzyme status; A positive association was found between LH and *SOD1, SOD2* gene, whereas a negative correlation was shown between *SOD1, SOD2* gene, and FSH hormone. These results provide supportive evidence that LH and FSH hormones in PCOS may contribute to enhanced antioxidant enzymes and oxidative stress.

Recommendations

1. In order to cover the entire physiological effect of the oxidative stress on the antioxidant status, this study recommends conducting experiments to investigate the role of antioxidant enzymes in PCOS women in different samples such as follicular fluids, ovarian and uterine tissue.
2. More molecular studies on *SOD1* and *SOD2* genes, including gene polymorphism and other medication that might be affected on gene expression and activity of Cu/ZnSOD, MnSOD enzymes in PCOS women need to be involved.
3. This study encourages the researchers to carry out more molecular and biochemical experiments on the other antioxidant enzyme such as catalase, glutathione peroxidase, and paraoxonase to present more knowledge about the role of enzymatic defense toward oxidative damage.
4. The uses of metformin drug as an excellent treatment for PCOS due to its role in lowering Oxidative stress and enhancing antioxidant enzyme activity and their expression.
5. The involvement of antioxidants in the treatment of polycystic ovarian syndrome may be effective as a secondary therapy to avoid oxidative damage and as a potential option for overcoming metabolic and reproductive issues linked to the polycystic ovarian syndrome

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