Investigating the Effect of Light on Circadian Rhythms and their Association with PCOS Using Animal Model

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DECLARATION

I, Umm E Farwa, hereby declare that the research work entitled *" Investigating the Effect of Light on Circadian Rhythms and their Association with PCOS Using Animal Model"* is entirely my own work. This work has not been previously submitted for assessment elsewhere. Any materials or information obtained from external sources have been appropriately acknowledged and referenced.

Umm E Farwa

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"Glory be to ALLAH and all praise be to ALLAH and there is none worthy of worship except ALLAH, and ALLAH is the Greatest. And there is no might or power except with ALLAH, the Exalted, the Great One."

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Umm E Farwa

DEDICATION

I dedicate this thesis to my family. I'm especially appreciative to my supportive parents, whose words of advice and push for persistence still reverberate in my ears. I also dedicate this work to my sisters who are my constant source of encouragement and support during the difficulties and challenges of graduate school and throughout life. Without the support of my family, I wouldn't be able to complete my project. I am grateful to my family, who have put their trust in me and taught me to try and work hard for the things I want to achieve. It is because of my family where I'm today otherwise I would be nothing without them.

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List of Abbreviations

PCOS	Polycystic Ovary Syndrome
L/L	Light- Light
L/D	Light- Dark
T	Testosterone
LH	Luteinizing Hormone
FSH	Follicle- Stimulating Hormone
AMH	Anti- Mullerian Hormone
IR	Insulin Resistance
GnRH	Gonadotropin-releasing Hormone
HPA	Hypothalamic-Pituitary-Adrenal
ACTH	Adrenocorticotropic Hormone
GDM	Gestational Diabetes Mellitus
IGT	Impaired Glucose Tolerance
T2DM	Type 2 Diabetes
CVRF	Cardiovascular Risk Factor
CVD	Cardiovascular Disease

SHBG	Sex Hormone Binding Protein
BMI	Body Mass Index
NIH	National Institute of Health
NHP	Non- Human Primate

Abstract

Polycystic ovarian syndrome (PCOS) is the most prevalent metabolic and endocrine disorder in reproductive women. Under environmental conditions of constant light and light pollution, individual's circadian rhythms are disturbed, which represents an emerging risk factor for PCOS, as it causes reproductive and metabolic changes in a body. In this study, a correlation between altered circadian rhythms and PCOS has been studied. In vivo-analysis of PCOS was performed by exposing Wistar Rats to constant light for twenty- four hours. The two L/L groups of rats were exposed to different light intensities i.e., 240W for 16 weeks and 600W for 8 weeks. In comparison, L/D animal models were kept under twelve-hour light and twelve-hour dark cycle. Following observations have been made when animal models were tested for behavioral analysis. Rats under constant light showed symptoms of anxiety and loss of memory when tested by Elevated Maze Test and Morris Water Maze Test with P values of <0.0001. It is followed by high concentrations of androgens with P value of <0.0006, gonadotropin hormones with P value of <0.0001, anti-mullerian with P value of <0.01, insulin with P value of <0.0002, and stress hormones cortisol with P value of <0.04 observed in the continuous light- induced animal models, as animal model showed evidence of disrupted circadian rhythms and PCOS. The histopathological analysis of ovaries, liver, and kidney showed that organs appeared damaged by the effect of environmental stress of constant light and PCOS. The current findings suggest that besides genetic and metabolic factors, PCOS can be caused by certain environmental factors which are prevalent in common population.

Keywords: Polycystic ovary syndrome, circadian rhythms, metabolic disturbance, endocrinology, light induce animal model

Chapter 1

Introduction

1 Introduction

1.1 Background

National Institute of Health Science (NIH) defines PCOS as the most prevalent endocrine disorder in women of the reproductive age, cause anovulation and hyperandrogenism, which occurs in approximately 10- 15% of population worldwide (Armanini et al., 2022). Women suffering from PCOS have also established metabolic disorders such as obesity, type 2 diabetes mellitus, and insulin resistance. A diagnosis of PCOS can be made if signs of oligomenorrhea, acne, hirsutism, IR, hyperandrogenism, and obesity are present in women. In addition, PCOS women suffer from infertility due to the presence of polycystic ovaries inside a body (Nautiyal et al., 2022). In addition to reproductive problems, PCOS is significantly linked to a variety of metabolic conditions, including glucose intolerance, hepatic steatosis, dyslipidemia, hypertension, and diabetes mellitus type II (T2DM) (Liu et al., 2017). PCOS is a chronic condition, and its effects on reproduction and metabolism have been widely studied across the lifespan, as it often presents a combination of hyperandrogenism and menstrual disruption in younger females. PCOS women usually suffered from central or abdominal obesity that exacerbate insulin resistance. These females are at increased chance of glucose tolerance impairment, T2DM, cardiovascular disorders, hypertension, and metabolic syndrome later in their lives (Teede et al., 2010). Studies have shown that besides psychological illnesses, women experiencing PCOS frequently display signs of poor self-esteem, despair, and impaired quality of life (Deeks et al., 2011). PCOS is a multidimensional illness, caused by the combination of genetic and environmental factors. Although previously genetic abnormality has been reported to PCOS development, there is currently no consensus on an established genetic marker for

the condition (Lowe & Reddy, 2015). Furthermore, proteome profiling of tissues that are important for pathophysiology sheds light on the periodic changes in the cell's proteome, as phenotypic plasticity has been attributed to organ specific epigenetic modifications, which might not be altered by genetic code are mostly accomplished by the process of adding or removing chemical groups in the chromatic structure (Feinberg,2007). Investigating epigenetic and genetic patterns with their effect on protein profile has led to the discovery of biomarkers and understanding molecular pathogenesis of PCOS. Along with prolonged anovulation and hyperandrogenism, four more clinical features have been introduced for the diagnosis of PCOS in women, which have also widened the scope of PCOS definition (Crespo et al., 2018). Figure 1 shows the pathophysiology of PCOS, including hormonal imbalance and hormones affected by continuous light exposure and contribute to PCOS (Manu, Thomson Soni, 2022).

Although the cause of PCOS remains obscure, numerous studies show that unbalanced lifestyle, genetic abnormalities, hormonal imbalance, and environmental stress can also cause PCOS (Chu et al., 2020). Among the environmental factors, circadian rhythm disruptions have recently drawn an attention to contribute to PCOS when trans- meridian flight or shift work alters the timing of the light-dark cycle and circadian clock goes out of synchronization with the surrounding physical environment. Several studies have demonstrated that women who worked nightshifts typically experienced irregular menstrual periods, which are frequently linked to metabolic syndrome, dysmenorrhea, glucose metabolism dysregulation, and IR that all are known risk factors of PCOS (S. Li et al., 2020). The pathogenesis of PCOS and effect of the altered circadian rhythms on the metabolic, reproductive, and irregular behavioral characteristics of women suffering with

PCOS are still unknown. Understanding these problems could aid in comprehending the onset and progression of PCOS in women with altered circadian rhythms (Chu et al., 2020).

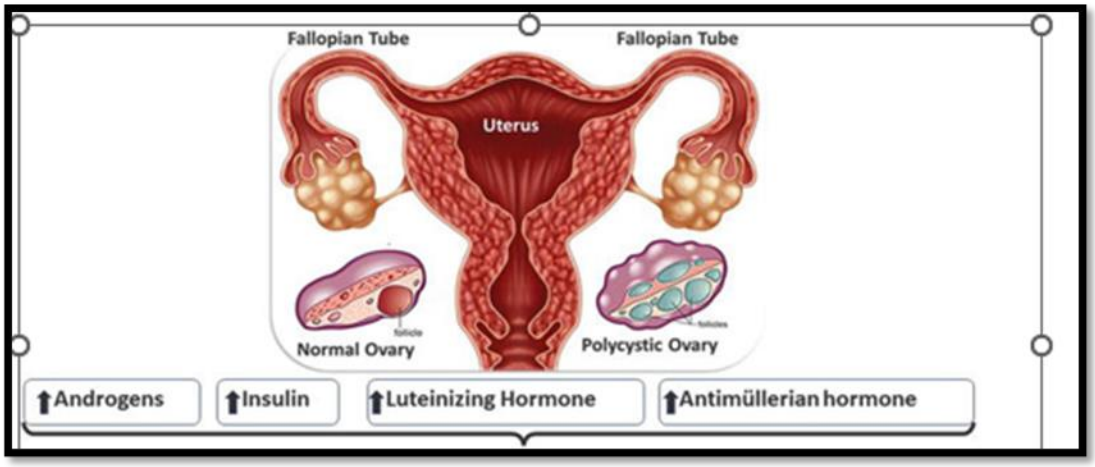


Figure 1 Pathophysiology of PCOS (Manu, Thomson Soni, 2022).

1.2 Role of Hormones in PCOS

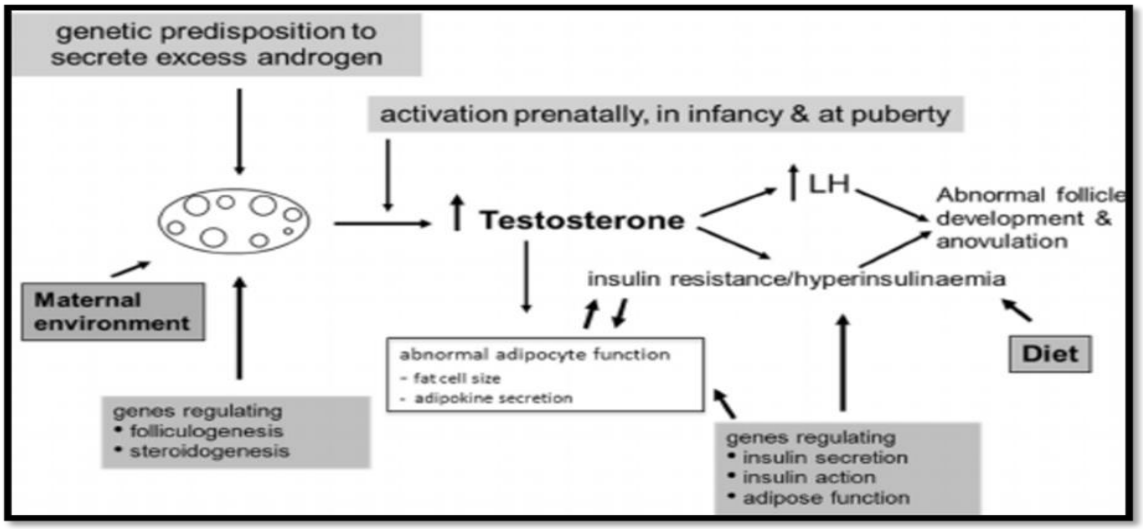
There are numerous hormones that play a role in growth and development of PCOS, including androgens like testosterone, LH, FSH, estrogen, progesterone, AMH, cortisol, and insulin, some of which are described below (Krishnan & Muthusami, 2017).

1.2.1 Testosterone

Women with PCOS have abnormal level of testosterone, a male sex hormone, in their blood, which ranges about 6.0- 86 ng/dl. The free testosterone, a quantity of testosterone that is physically active and unbound in the body, is also reported in minute quantities (0.7 and 3.6 pg/ml) in female's blood. Frequently, higher levels of both free testosterone and total testosterone are found in PCOS women. Furthermore, a woman's body's naturally occurring menstruation and ovulation can be suppressed by even a minor rise in testosterone levels (Krishnan and Muthusami 2017). Figure 2 shows an increase in

androgen synthesis, testosterone, due to environmental and genetic factor, as well as hormonal imbalance caused under stress environment, which is the basic indicator of PCOS (Franks, 2012).

Figure 2 Suggested function of testosterone in the developmental basis of PCOS (Franks,2012).



1.2.2 Luteinizing Hormone and Follicle-Stimulating Hormone; LH: FSH Ratio

In PCOS, FSH levels are usually normal or low in contrast with LH. This results from an unbalanced feedback loop among the ovaries, pituitary gland, and hypothalamus (Georgopoulos et al., 2010). Gonadotropin-releasing hormone (GnRH), which is normally produced by the hypothalamus in the brain, induces the pituitary gland to secrete LH and FSH, which then promote follicle growth and development in ovaries and ultimately results in ovulation (Malini & George, 2018). However, an excess of estrogen and androgens is frequently present in PCOS, which might interfere with the feedback system. High androgen levels limit the production of hormones resulting in low levels of FSH. In

addition, elevated levels of estrogen affect the hypothalamus to secrete lower levels of GnRH that further decreases the synthesis of FSH (Y. Xu & Qiao, 2022).

On the other hand, the feedback loop may be disrupted by the high levels of androgens that the ovaries produce in PCOS, which will result in the pituitary producing more LH than FSH, as a result, the ratio of LH to FSH (2:1, or even higher 3:1) is imbalanced, with LH to be higher than FSH (Malini & Roy, 2021). This hormonal imbalance between the LH and FSH in the PCOS women can lead to higher production of androgens, which cause disrupt menstrual cycle, lack of ovulation, and cyst development in ovaries (Saadia, 2020a).

1.2.3 Anti- Mullerian Hormone (AMH)

Recently, a relation between PCOS and serum AMH has been implicated as a key diagnostic sign in the PCOS pathophysiology based on higher serum AMH levels in PCOS. Similar to inhibin and activin, AMH is a protein that belongs to TGF-β family formed by the granulosa cells of ovaries and plays a fundamental role in the follicle growth inside ovaries (Knight and Glister 2006). The concentration of AMH is high during the developing stage of follicles, and as they mature the levels gradually decline (Sahmay et al. 2013). In PCOS women, higher levels of AMH are related to the increased immature follicles found in ovaries, which results in anovulation, irregular menstrual cycle, and infertility (Stracquadanio, Ciotta, and Palumbo 2018).

Many studies have been conducted on the correlation between PCOS and AMH levels, which indicated that females with PCOS have greater AMH levels than those without the disorder. According to the research issued in journal of Clinical Endocrinology and

Metabolism, PCOS women have two to three times the amount of AMH as those without the disorder (Moolhuijsen et al. 2022). The higher levels of AMH in PCOS patients have consequences for their reproductive health. AMH has now been demonstrated in animal models of PCOS to play a potential role in the onset of the condition by exposing fetus to high amounts of the hormone in utero (Tata et al. 2018). In females, AMH suppresses the migration of primordial follicles from the resting oocyte pools and may inhibit the action of FSH which can cause ovulatory abnormalities (Pigny et al. 2006). Overall, serum levels of AMH are higher in women suffering with PCOS compared to the women who are ovulating normally (Sirotkin and Grossmann 2015) (Bertoldo et al. 2014). Figure 3 shows an increase in AMH in PCOS, which further disrupts LH: FSH ratio causing an increase in LH and decrease in FSH, ultimately leading to hyperandrogenism (Bhattacharya et al., 2022).

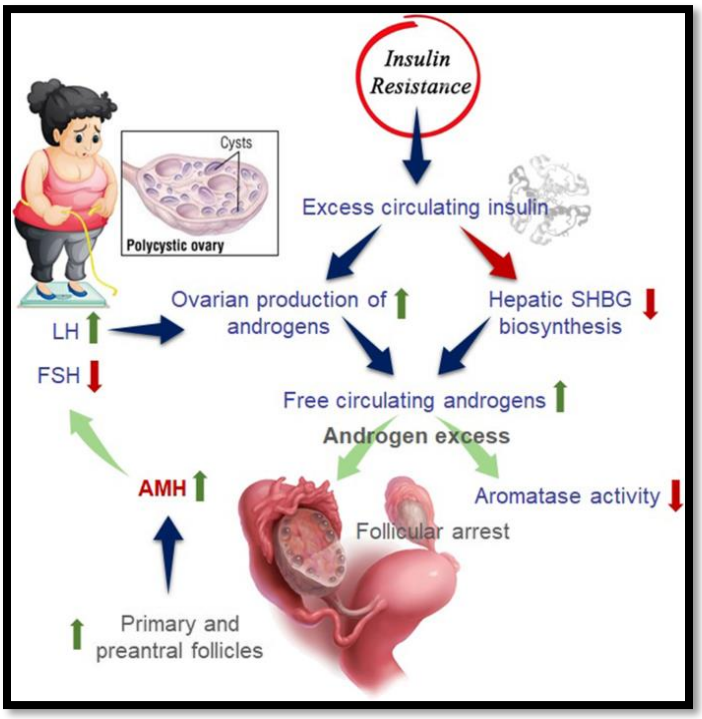


Figure 3 Role of Anti- mullerian hormone in pathophysiology of PCOS (Bhattacharya et al., 2000)

1.2.4 Insulin Hormone

According to studies, insulin resistance affects up to 70% of females with PCOS but only 10% of those without the condition, developing a close relationship between IR and PCOS (Moghetti & Tosi, 2021). Although many studies have been conducted, the precise mechanisms underlying the link between IR and PCOS remain to be completely understood.

Figure 4 suggests that IR results in hyperinsulinemia, in which an insulin excess is found in bloodstream, which further results in an increase androgen synthesis by ovaries, and hirsutism, forming PCOS- like symptoms (J. Wang et al., 2019). Moreover, hyperinsulinemia can enhance LH synthesis, which further stimulates ovaries to synthesize more androgens (Y. Xu & Qiao, 2022).

Another study suggested that IR causes levels of insulin-like growth factor 1 (IGF-1) to increase. IGF-1, a hormone that shares structural similarities with insulin and is likely essential for regulating glucose metabolism. IGF-1is a significant activator of androgen synthesis by ovaries, is a significant activator of androgen synthesis (Stepto et al., 2019).

The development of IR in PCOS is influenced by numerous factors. One of the most significant causes is obesity, as IR can result from the cytokines and hormones produced by adipose tissue, which can disrupt insulin signaling (Stepto et al., 2020).

In addition to excess body fat, hormonal imbalances, inflammation, and genetic predisposition also contribute to IR lead to PCOS (Zeng et al., 2020). While there is evidence of familial link, genetic factors may also contribute to the development of IR in PCOS.

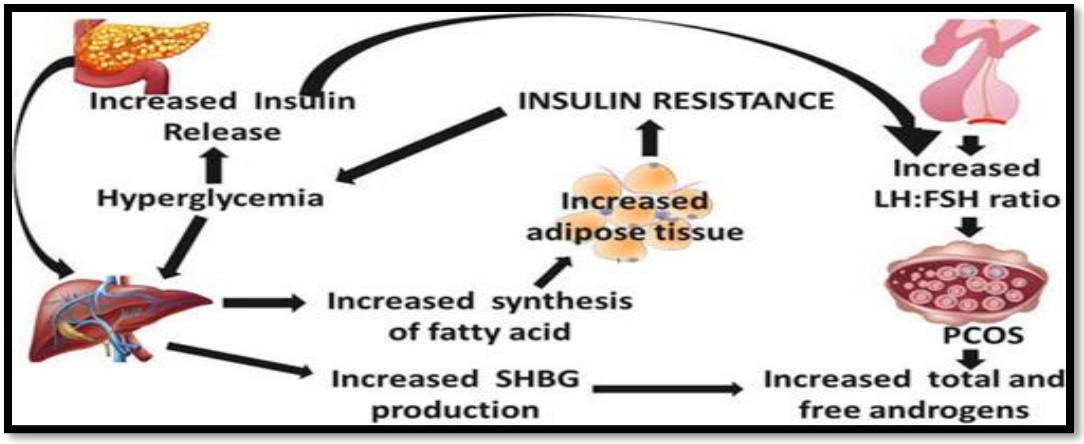


Figure 4 Role of Insulin in pathophysiology of PCOS (Y. Xu & Qiao, 2022).

1.2.5 Cortisol Hormone

Cortisol is a hormone that plays an essential role in a stress response. It is secreted by the two adrenal glands, one of which is located on top of each kidney. The right balance of cortisol is an essential element for health, and its imbalance can have negative effects on the body. However, under stress conditions, such as constant light exposure, more cortisol is released into blood circulation and causes reproductive and metabolic problems. Previous research has revealed that in POCS the hypothalamic-pituitary-adrenal (HPA) axis is more active, and that cortisol secretion is higher, which further increases the adrenal androgen due to the excessive adrenocorticotropic hormone (ACTH) secretion (Benjamin et al., 2021).

1.3 Normal Ovary vs PCOS Ovary

Traditionally, egg cells are stored in ovarian follicles and released during ovulation. Abnormalities in hormone levels inhibit follicles from developing and maturing to produce egg cells in polycystic ovary syndrome, instead these immature follicles gather in the

ovaries. Women who are affected may have 12 or more of these follicles. In PCOS, the right ovary is slightly bigger than the left ovary. Recent research in PCOS women showed the presence of more than follicles in ovary. The cause of these variations in ovaries is obscure (S. Li et al., 2020). Some studies also found that ovaries in PCOS have greater volume with large number of antral- follicles present in them as compared to the normal ovaries (Chu et al., 2020).

1.4 Problem Statement

With the help of animal models that are exposed to constant light, this study aims to examine how circadian rhythm disruption affects reproduction, metabolism, and behavior. In addition, it attempts to investigate the connection between PCOS and circadian rhythms.

1.5 Aims and Objectives

The aims and objectives of this research project are following:

i. Evaluation of the effect of disruption of circadian rhythms by constant light exposure on reproduction and metabolism by inducing PCOS in animal models.
ii. Evaluation of behavioral changes in response to the change in estrogen cycle due to continuous light- induced PCOS.
iii. Histopathological Analysis of tissue damage due to the continuous light- induced PCOS.

Chapter 2

Review of Literature

2 Review of Literature

2.1 Polycystic Ovary Syndrome (PCOS)

PCOS is the most prevalent endocrinopathy and happens to be least understood. It is thought to affect 4% to 20% of women in their reproductive age worldwide (Dennett & Simon, 2015), and is categorized by hyperandrogenism, oligo-/anovulation, and polycystic ovaries (Chu et al., 2020). It is a syndrome where ovaries create high levels of male sex hormones known as androgens, which are typically present in women in small amount. Polycystic syndrome refers to the condition in which ovaries develop many tiny cysts (fluid- filled sac). Moreover, it is linked to a variety of metabolic, reproductive, and behavioral (stress, memory loss, and eating disorder etc.) features, including diabetes GDM, IGT, T2DM, CVRF, and CVD. Women with the condition is typically seen with the insulin resistance, which is a hallmark of PCOS and key factor in causing metabolic and reproductive issues in PCOS. Moreover, environmental variables including diet and nutrition, environmental toxins, geography, and socioeconomic status are also thought to contribute to the chronic persistent hyperglycemia that leads to the proinflammatory state found in PCOS patients. There is some evidence that environmental contaminants might affect reproductive health, but little is known about how they may influence the onset of PCOS (Kakoly et al., 2019).

Number of studies show that genetic mutations, environmental factors, and hormonal imbalance, along with unhealthy lifestyle contribute to PCOS. The formation and progression of PCOS are significantly influenced by distorted circadian rhythms brought on by environmental stress, which has attracted attention recently. When trans meridian

flight or shift work alters the L/D cycle, the circadian system is typically out of synchronization with the surrounding physical environment, which has a negative impact on health. Many research findings showed that women who work night- shifts and expose to uneven L/D condition have irregular menstrual cycle, which is mostly associated with dysmenorrhea, IR, and GDM that are also known as risk factors of PCOS. Animal models like rodents were exposed to constant light was reported to develop hyperandrogenism and PCOS demonstrating the interlink between disrupted circadian rhythms and PCOS manifestation. However, it is still unknown how circadian rhythm disturbance affects PCOS's reproductive symptoms and what causes this etiology (Chu et al., 2020). Understanding these problems could aid in comprehending the onset and progression of PCOS in women with circadian rhythm disturbances. In this review, we investigate the role of circadian rhythm disruptions to determine their effect on manifestation of PCOS with evidence gaps and suggest future studies.

2.2 Etiology of PCOS

Etiology of PCOS is complicated caused by genetic factors, environmental stress, and their combination. It includes many features, involving dysfunctional hypothalamic pituitary, hyperandrogenism, increase gonadotropin secretion, IR, and anovulation. The primary contributors to the underlying hormonal imbalance seen in women with PCOS are insulin resistance and hyperandrogenism. The pathophysiology of PCOS is influenced by the interaction of these risk factors, such as unbalanced life with genetic mutations and environmental stress (Kakoly et al., 2019).

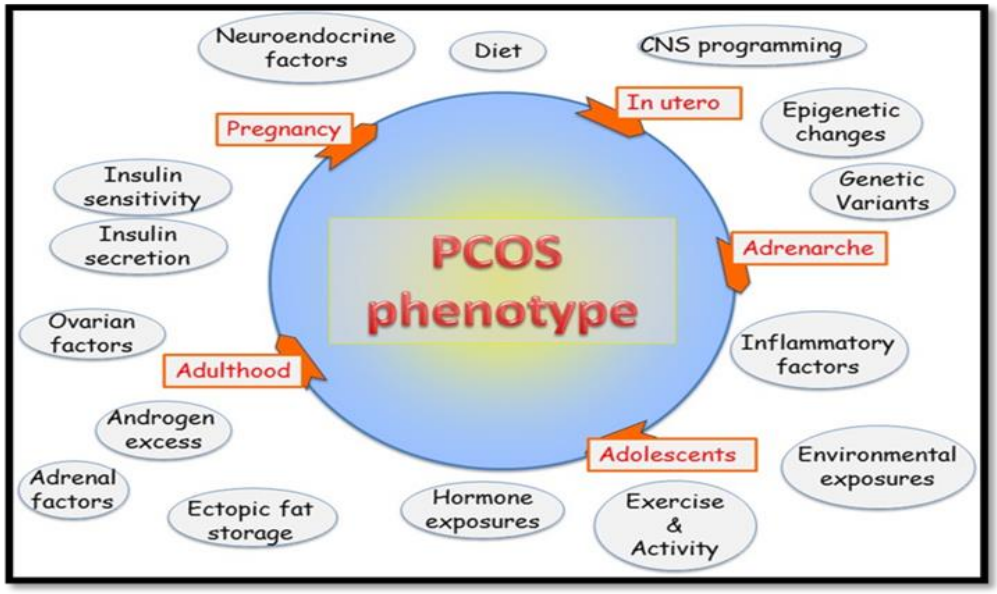


Figure 5 Phenotype of PCOS related factors women's entire lifecycle is affected by PCOS. Circular symbols depict variables that could have an impact on PCOS pathogenesis. A person's response to various factors varies. PCOS is the biological network's representative of interconnected neuroendocrine, hormonal, metabolic, genetic, and environmental factors.

2.2.1 Insulin Resistance and Hyperandrogenism

Hyperandrogenism occurs in about 60-80% of women suffering with PCOS. The two prime sources of androgens are ovaries and adrenal cortex, which in the condition release androgens to high levels. Evidence collected through experimentation has indicated that early androgen exposure at an early developmental phase may have a negative impact on a subsequent reproductive and metabolic changes in women suffering with PCOS. Elevated androgen levels are known to cause certain cardiometabolic disorder (Kakoly et al., 2019). Hyperandrogenism refers to the disorder that occurs when the levels of male hormone androgen rise above the normal range in females. In polycystic ovary syndrome ovarian and extraovarian hyperandrogenism are considered a major symptom. The production of

androgen in the ovary's theca cells contributes to the hyperandrogenic state in the ovary. It is noted that the concentration of testosterone will be significantly higher in the disease than in the normal. Although testosterone is a male sex hormone, women also produce small amount of it as part of regular metabolic activity. Normally, the levels of androgens produced by the ovaries and adrenal glands in females are approximately equal. Increased levels of ovarian androgen are thought to be a common sign of hyperandrogenism in PCOS, which impairs follicular maturation as increased levels of androgens would likely to negatively affect the follicular growth and cause atresia. In PCOS, the ovaries are generally believed to be the main cause of androgen excess, but it has been discovered that 20–30% of patients have elevated levels of adrenal androgen. According to certain scientific studies, there is a correlation between the ovarian androgen and adrenal androgen in PCOS, as excess of ovarian androgen leads to elevated adrenal androgen. Despite adequate research, adrenal hyperandrogenism could not be investigated at the molecular level due to the hazards associated with collecting the patients' adrenal glands. In addition to this, hyperandrogenism may also lead to oxidative stress and persistent inflammation linked to PCOS.

The etiology of PCOS is also significantly influenced by the IR associated with hyperinsulinemia and is found in about 75% lean women and 95% overweight or obese women that are also suffering with PCOS. IR is decrease in the ability of insulin to perform metabolic functions of glucose uptake and synthesis, as well as lipolysis. As a result, increased insulin is required to perform a particular metabolic function. Hence, IR is described by the elevated levels of blood insulin, at both stages during rest and glucose load considering β-cell function of pancreas is still present. Insulin functions to boost LH

induced androgen synthesis in the theca cells of ovaries and GnRH-mediated gonadotropin secretion. Additionally, insulin raises the number of bioavailable androgens by decreasing the synthesis of hepatic SHBG (sex hormone binding globulin). Results from recent meta-analysis, which evaluated insulin function in PCOS patients by using the euglycemic hyperinsulinemia insulin clamps of gold standard, have supported an idea of relationship between insulin resistance and PCOS with patients exhibiting 27% reduction in blood insulin action regardless of BMI. These results imply that IR is a fundamental aspect of PCOS. There is a definite need for more study to advance our understanding of the entire etiology of PCOS because we are still far from it.

2.2.2 Arenal, Ovary and Androgen Excess

The hallmark of PCOS is an excessive synthesis of androgens by ovaries and adrenal gland. The abnormally high levels of insulin production are caused by both internal and external factors such as steroidogenesis and hyperinsulinemia. In contrast to normal women, women with PCOS have more developing follicles and early growth arrest of antral follicles at 5-8mm. The irregular interactions between paracrine, endocrine factors, and autocrine factors, which are critical of follicle growth and maturation, can contribute to the dysregulated function of ovaries in PCOS patients (Witchel et al., 2019).

In the follicular maturation, forming during gestation, primordial follicles are composed of meiotically arrested oocytes enclosed by pregranulosa cells. Therefore, women ovaries are exposed to the ambient maternal environmental conditions throughout the pregnancy. Till the initiation of menstrual cycle ovaries are comparatively dormant, however detailed analysis of follicle morphology in ovaries that are at prepubertal, or early pubertal stages

is insufficient. Moreover, the accurate pathways that are involved in activation of follicle growth are poorly known (Witchel et al., 2019).

A glycoprotein, AMH, suppresses initial follicle activation and signifies follicle reserves, but when AMH suppresses antral-follicle growth and maturation it appears to stimulate the growth of preantral-follicle to antral-stage in NHP ovaries, as high levels of AMH is found in antral- follicles (Witchel et al., 2019).

2.2.3 Neuroendocrine Factors

Women with PCOS have been found to have higher LH pulse frequency, amplitude, and LH/FSH ratios. The initial symptoms of PCOS appear during the start of pubertal growth along with increased secretion of gonadotropin hormones, estrogen, and reactivation of the hypothalamus gonadotropin hormone pulse generator. Loci found in the genome wide association involve FSH-β polypeptide genes, FSHR, and LHCGR highlighting neuroendocrine roles to PCOS pathogenesis (Witchel et al., 2019).

Hypothalamic neurons present in nucleus release the neurokinin B, dynorphin, and kisspeptin, which are identified as likeable candidates for the hypothalamus GnRH pulse generator. The GnRH neurons and GnRH pulse generator instead of initiating puberty, served as downstream nodes influenced by neurosecretory factors and some other hormones. In other words, the output came through the generator of GnRH pulse is regulated by the stimulation of the excitatory inputs, as well as by the deactivation of the inhibitory inputs, modulated by variety of factors, to control GnRH is secreted in the form of the isolated pulses, which travel throughout the structure of median eminence to the gonadotrophs of pituitary and result in pulsatile secretion of LH and FSH. The pulse

frequency of GnRH modulates the pulse frequencies of LH and FSH. LH pulse frequency rises with increased GnRH pulse frequency, but FSH pulse frequency falls. The elevated pulsatile GnRH production is probably what causes the increased LH pulse amplitude and pulse frequency seen in PCOS (Witchel et al., 2019).

Gonadotropin releasing hormone (GnRH) neurons exhibit estrogen receptor-β, but don't exhibit estrogen receptor-α, progesterone, or androgen receptor. Therefore, negative feedback mediated by the steroids is not direct rather it is generated through neuronal network of hypothalamus present upstream of GnRH neuron, which mechanism is impaired in PCOS that requires higher estradiol and progesterone concentrations the timing of puberty. As a result of this process, gonadotropin and GnRH secretion are elevated (Witchel et al., 2019).

2.2.4 Nutrient Excess and Obesity

Obesity or overweight common in young girls and full- grown women is responsible for PCOS. Excess nutrients enable adipocytes to go bigger in size, a condition known as hypertrophy, or produce additional adipocytes, a condition known as hyperplasia. According to this enlargement theory, hypertrophy forms microenvironments identified by the secretion of proinflammatory cytokines, hypoxia, as well as release of free-fatty acids, invasion of macrophages, and insulin resistance. Insulin resistance reduces inhibition of adipocyte lipolysis, which enhances the serum triglycerides and free fatty acid eventually leading to increase in hyperlipidemia and hepatic de novo lipogenesis. Data collected from females of normal weight but suffering with the disorder indicated the increase in the mass of abdominal fat, which is due to the deposition of the intraabdominal fat, along with enhanced abdominal adipocytes population. Another study found that adolescent girls with

PCOS have increased concentration of hydroxyprogesterone, decreased sensitivity of insulin, and suppression of insulin-induction of the non-esterified fatty acids when compared to the girls without the disorder. Another small study by using regular samples of the IV-glucose-tolerance tests revealed the early dysfunctioning of cells in first-degree female relatives of PCOS-affected women who were overweight or obese as opposed to control girls who were overweight or obese. The conclusions that may be derived from these investigations are constrained by the small sample sizes. However, some findings suggest that girls who are “destined" to have PCOS are diagnosed with altered cellular function and insulin sensitivity, which might have started in childhood or during early stage of adulthood. Enzymes that activate and inactivate androgen precursors are expressed in adipose tissue. Aldo-ketoreductase type 1C an enzyme encoded by the gene AKR1C3 is expressed by adipose tissue converts androstenedione (pre- androgen) into testosterone (androgen). In addition, SRD5A1 gene encodes 5'-reductase type 1, an enzyme expressed in adipose tissue converts testosterone to DHT. In women with PCOS, subcutaneous fat had higher AKR1C3 and lower SRD5A1 mRNA expression, according to the in-vivo metabolic phenotyping analysis (Witchel et al., 2019).

Girls with obesity and PCOS were a group of people in which the effect of androgen on the lipid metabolism was described. This category showed reduced mobilization of lipids, oxidation of fats, and impaired conversation of lipids into carbohydrates, along with stimulation of insulin as compared to those without the disorder (Witchel et al., 2019).

2.3 Difficult Genetics of PCOS

The idea that PCOS is a heritable disease is supported by research demonstrating ethnic propensity, familial aggregation, concordant twin studies, and relationship with other

Mendelian disorders. The evidence that disease aggregates in families forms the basis of genetic studies. Though familial clustering may often have environmental causes, there has been no proof of infectious agent or environmental disruptor to cause PCOS. The difficulty in establishing the parental phenotypes, and/or the uncertainty surrounding the mal phenotype resulted in the non-availability of family studies of PCOS that would demonstrate a mechanism of inheritance. Due to insufficient data present on family studies of PCOS, none of the inheritance modes are conclusively established. Despite the diversity in study design and the difficulties to get sufficient phenotypic data to allow a formal segregation analysis, the present research clearly indicates that PCOS tends to cluster in families with an autosomal dominant pattern of inheritance (Diamanti-Kandarakis et al., 2006).

2.3.1 Genetic Mutations in PCOS

To identify genetic mutations that are inherited and cause PCOS, various research analysis has been conducted, but no single gene mutation's real penetrance has been reported to this date. All genes or mutations found in a familial aggregation has a poor penetrance, hence must be combined with additional covariates, hormonal or environmental variables to result in disease. In conclusion, the syndromic condition PCOS is multifactorial and polygenic.

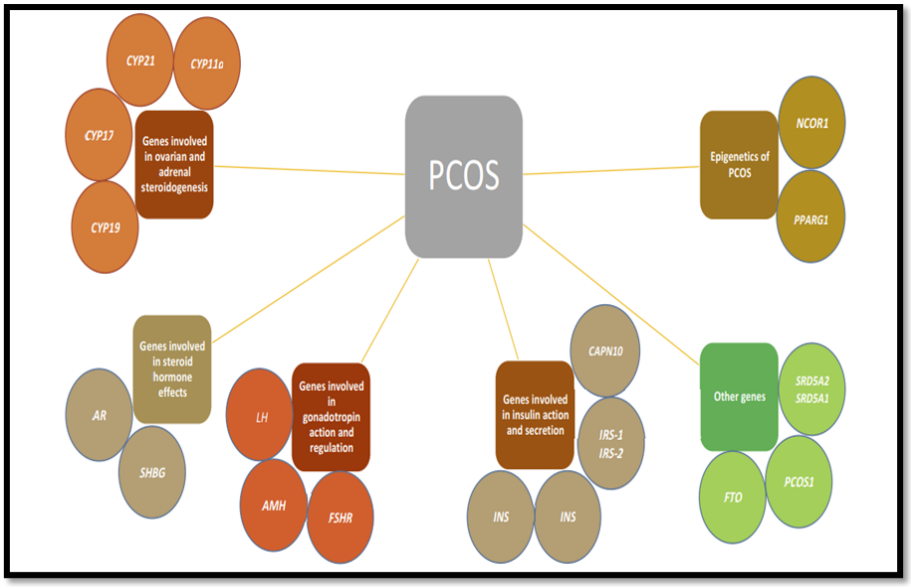


Figure 6 Overview of genes involved in the PCOS indicating multifactorial characteristic of the disease.

2.3.2 Genetic Mutations Linked to PCOS

PCOS is a complex disorder caused by many genetic abnormalities. The overview of genes involve in PCOS is given below:

2.3.3 Genes Involved in PCOS

The endocrine factor that is most common with PCOS is increased androgen level. Therefore, several genes associated with elevated levels of androgen linked to PCOS have been identified, as discussed below.

CYP11a

The CYP11a gene expressed an enzyme that is necessary for a transitional stage in the conversion of cholesterol to progesterone. This phase of the conversion of cholesterol is rate-limiting (Franks et al., 2000). Furthermore, studies also reported variations and polymorphs associated with PCOS.

CYP21

The gene CYP21 encodes an enzyme that catalyzes the conversion of 17-hydroxyprogesterone to 11-deoxycortisol during the formation of steroid hormones. A far less active enzyme because of variations results in inefficient anabolism steroidogenesis, which further contributes to PCOS (Witchel & Aston, 2000). Witchel identified CYP21, a heterogeneous gene, linked to the hyperandrogenemia PCOS- like illness. However, a direct link between CYP21 and PCOS has not been found (Witchel et al., 2005).

CYP17

CYP17 encodes an enzyme that catalyzes the conversion of progesterone and pregnenolone into 17-hydroxyprogesterone and 17-hydroxypregnenolone, respectively. Studies indicated that the theca cells of ovaries produce an increased expression of the gene CYP17. (Wickenheisser et al., 2000). Scientists also identified polymorphs in the promoter region linked to PCOS.

CYP19

Located on chromosome 15q21.2, CYP19 expresses aromatase p450, necessary for production of estrogen (Takayama et al., 2004). Both obese and underweight PCOS women have been observed to have lower aromatase activity.

2.4 Epigenetics of the PCOS

Epigenetic changes are heritable variations in the expression of genes that are not resulted from changes in DNA sequence but are transgenerational and inherited mitotically. According to reports, numerous disorders, including T2DM, PCOS, and prostate cancer,

are influenced by epigenetic factors (N. Xu et al., 2010) (Ho et al., 2006). Increased secretions of androgens in prenatal development have been investigated to cause illness in monkeys, sheep, and rat models. The symptom of the disorder is very much alike to PCOS (Wang et al., 2012) (Abbott et al., 2009). Studies have indicated that higher levels of androgen in fetal development predispose the offspring to PCOS- like symptoms in later stages. Qu et al. identified a hyperandrogenism-induced epigenetic modification that leads to ovarian failure caused by the variant CPG island- methylation in the gene PPARG1 and NCOR1 gene expressed by granulosa cells (Qu et al., 2012). Ning Xu identified a distorted pattern of DNA methylation in the chromosomes of PCOS patients compared to the heathy individuals (N. Xu et al., 2010). This epigenetics role of PCOS indicates multifactorial aspect of the disease.

2.5 Inheritance of PCOS

PCOS is a compound disorder that affects a considerable number of people worldwide. People with PCOS experience numerous social and stress-related problems, hence, different aspects of the condition were investigated to draw a firm conclusion. In ancient times, in 1972, infertile females with small ovaries were recognized (Insler & Lunenfeld, 1990). Another investigation into the degenerating ovaries was described later, in 1844 (Chéreau, 1844). Research continued several factors of PCOS, including its cellular mechanism, genetic predispositions, hormonal involvement, and environmental risk aspects. Cooper and colleagues published the first study on the genetic basis of PCOS in 1968. PCOS studies in families have documented multiple relatives with an inheritance of autosomal dominance. The autosomal dominant inheritance theory for PCOS was supported by the fact that about 55–60% of the first-degree relatives of the proband had

the condition. Later, single-gene causes of male-pattern baldness, oligomenorrhea in PCOS women, and hirsutism were discovered (Khan et al., 2003). Twin studies in short populations of monozygotic and dizygotic twin pairs revealed that PCOS is an X-linked polygenic disorder rather than an autosomal dominant or monogenic disease (Joharatnam et al., 2011) (Rocha et al., 2011). Furthermore, twin studies indicated that genetics played a role in 72% of the diversity in PCOS risk, underscoring the genetic component (Vink et al., 2006).

2.6 Incidence and Prevalence of PCOS

The National Institutes of Health (NIH) established criteria for PCOS diagnosis in 1990, and the assessment of PCOS prevalence relied on how many of these criteria were met. According to estimates, the prevalence of PCOS is 4.0% in both Caucasians and Black people, who make up the majority of the population (Singh et al., 2018) (Kiconco et al., 2021). In later investigation, including 400 women, it was predicted to be 6.6% in both age groups (18- 45), however a clear difference were seen between white (8%) and black women (4%) regarding the prevalence of PCOS (Chan et al., 2017). The estimated prevalence among Greek women 6.8% of females seeking assistance at free medical camps. A study of Caucasian Spaniards estimated the 6.8% is the prevalence (Nandi et al., 2014) (Chan et al., 2017). Oxford University Research Facility and Private Medical Facility reported an incidence of PCOS of 6.8%. About 5.6% of the Adult Chinese Women is at the risk of developing the disease. Nearly all other populations share this prevalence (R. Li et al., 2013). Additionally, almost 9.13% of Indian women of reproductive age have PCOS (Sucato et al., 2011). Compared to Caucasians, South Asia Population, particularly Pakistanis, has much higher frequency of developing the disorder. According to Rotterdam

criterion 2003, Akram and Roohiet observed greater chances of PCOS, approximately 50% in Asians (Akram & Roohi, 2015). In a similar manner, Zahida et al. (2010) found that 40% of infertile women in Karachi, Pakistan, seeking medical treatment have PCOS (Baqai et al., 2010).

Anovulation and hyperandrogenism are the clinical hallmarks of polycystic ovarian syndrome, which has a prevalence of 4-12% and affects around 5% of women of reproductive age. PCOS can be identified in up to 10% of women during gynecological exams. Depending on the criteria used to make the diagnosis, the prevalence of PCOS can range from 5% to 20%, although it can reach 15% to 20% when the American Society for Reproductive Medicine/European Society for Human Reproduction and Embryology criteria are applied.

Women in South Asia, including 52% of Pakistani women, have higher rates of PCOS. The prevalence of PCOS in Pakistan has rapidly seen a surge effecting adolescent girl between 9% and 17%. In India, the prevalence of PCOS varies from 3.7 to 22.5% depending on the population investigated and the diagnostic criteria applied.

2.7 The Diagnostic Dilemma of PCOS

PCOS has typically been diagnosed based on a history of oligomenorrhea or hyperandrogenism, which can be biochemical (elevated circulating total or bioavailable androgens) or clinical (most often hirsutism) in nature. Additionally, polycystic ovaries may also be present. The "consensus" definition left out the polycystic ovary morphology, which is most frequently identified on ultrasonography today and consists of many 2–8 mm subcapsular preantral follicles and an enlarged ovarian volume. But these polycystic

ovary ultrasound parameters are often a changing target. With the exclusion of secondary causes like hyperprolactinemia and non-classical congenital adrenal hyperplasia, the Rotterdam criteria, which includes any two of the three signs of persistent hyperandrogenism, anovulation, and polycystic ovarian structure on ultrasound, should be used to diagnose PCOS (Diamanti-Kandarakis et al., 2006). Hyperandrogenism can be identified both biochemically and clinically. Biochemical hyperandrogenism may be confirmed by laboratory tests that show an increase in the free- testosterone, FAI, or the bio-available testosterone. The morphology of PCOS should be comprised of more than 20 follicles in ovaries, or the ovarian volume should be more than 10ml, when detected through ultrasound. However, it's crucial to remember that in women who have both hyperandrogenism and oligomenorrhea ultrasound evidence of PCOS may be necessary for the diagnosis, as it is not advised for diagnosis in teens (Kakoly et al., 2019).

2.8 Clinical Picture of PCOS

PCOS the title suggests a disorder that affects ovaries and formation of cysts. It is due to the hormonal imbalance that s further marked by irregular menstrual cycle, causing several ovarian cysts, hirsutism, and amenorrhea in women of reproductive age. PCOS is a complex disease, primarily results in infertility, and hence develops social imbalance. PCOS patients have high amounts of androgen, which results in subcellular abnormalities in the theca cells. Despite the lack of trophic factors, theca cell steroidogenesis is intrinsically activated in PCOS patients' theca cells, resulting in the secretion of a high level of androgen (Cadagan et al., 2016). In various studies, increased pre-antral and low antral follicle numbers have also been identified in PCOS. Women with PCOS have more follicles overall due to a faulty apoptotic mechanism in maturing follicles (Das et al., 2008). The

malfunction in insulin signaling pathway is also due to abnormality of PCOS. Similarly, mutations in gene expression of insulin signaling pathway have also been linked to PCOS. The other mechanisms such as glycol-oxidative stress are also reported as an important part of PCOS pathophysiology. IR can be resulted from oxidative stress as well, which further results in hyperandrogenism (Victor et al., 2009). As its name suggests, it is a complicated illness with syndromic pathology. It has multiple causes and frequently exhibits a range of symptoms. The four phenotypes of the illness are outlined below.

2.8.1 Classification of PCOS Built on Phenotypes

PCOS has four different phenotypes, including phenotype A, B, C, and D. Generally, hyperandrogenism, menstrual irregularity, and BMI are termed as independent risk factors of PCOS. It is further grouped in four variable phenotypes.

i. Hyperandrogenism, polycystic ovarian structure, and anovulation.

ii. Hyperandrogenism, Normal ovaries with Anovulation condition

iii. Hyperandrogenism and ovaries with regular menstrual cycle

iv. Polycystic ovaries and anovulation

Phenotypes A and B

These phenotypes are termed as standard PCOS models. PCOS A and B patients exhibit irregular menstrual cycle, increased secretion of insulin, and high risk of metabolic diseases. It also shows symptoms of atherogenic dyslipidemia (AD) and obesity (Vickers, 2017). In contrast to other phenotypes having a normal amount of androgen in blood, more cases of hepatic steatosis have been observed with PCOS phenotypes A and B (Goverde et

al., 2009). Additionally, the anti-mullerian hormone is markedly raised in the classic PCOS (Hashemi et al., 2014).

Phenotype C: Ovulatory PCOS

Phenotype C in comparison to classic and non-hyperandrogenic form of PCOS, phenotype C (ovulatory PCOS) women commonly have mildly raised levels of serum insulin, androgen, and atherogenic lipids, as well as high hirsutism levels. Metabolic syndromes are also prevalent in PCOS C type (Mitchell et al., 2011).

Phenotype D: Non- hyperandrogenic PCOS

Phenotype D is typically characterized by low metabolic dysfunction, normal testosterone levels, and slightly raised levels of other endocrine hormones (Dewailly et al., 2006) (Pavičić Baldani et al., 2013). The endocrine study of PCOS phenotype D shows higher levels of SHBG, lower levels of testosterone types T3 and T4, as well as lower ratio of LH to FSH (Jamil et al., 2016). Normally women with such PCOS have regular period cycle (Tsatsanis et al., 2015).

2.9 Circadian Rhythm Alteration as a Novel Pre-Requisite For PCOS

The circadian clock, a significant environmental risk factor that enables animals to regulate their behavior and biology, is fundamentally involved in many diseases. Each tissue's circadian clock coordinates internal and external inputs to control the synthesis of genes of circadian clock in a way that is peculiar to that tissue. An increasing number of studies show that interference with the circadian clock has increased the risk of metabolic and endocrine disorders. According to recent studies, early misalignment of circadian rhythms is linked to insulin insensitivity and high levels of blood testosterone in PCOS. Humans

have been shown to develop insulin resistance because of jet lag and shift employment. In rats, light interference in dark period raises blood glucose, which ultimately increases insulin. On other hand, increase androgen synthesis by ovaries is consider as the primary hallmark of PCOS, and high concentration of testosterone is the most pervasive hormonal abnormality of PCOS. After prolonged exposure to light, rats show higher serum androgen levels. Circadian clock genes are directly related to serum free and SHBG bound testosterone. PER2 expression changes in conjunction with the synthesis of testosterone, which was found to be higher in morning indicates close connection between androgen synthesis and PER2 expression. Additionally, increased granulosa cell death is a significant factor in PCOS patients' ovarian dysfunction as well as the follicular atresia that results in female infertility. The current study aims to establish the potential role of the altered circadian rhythms and their association with PCOS (S. Li et al., 2020).

2.10 Current Treatment of PCOS

PCOS cannot be cured, but there are therapeutic options available to help reproductive women. The treatment modalities for PCOS are mentioned below in detail.

2.10.1 Dietary Therapy

30% of PCOS individuals are diagnosed with obesity. Dietary therapy is one of the effective modes through which symptoms of PCOS can be reduced, including insulin resistance and irregular menstrual cycles. Since dieting habits and exercise do not demonstrate long-term results, Bariatric surgery has been adopted to obtain more hopeful results (Sjöström et al., 2007).

2.10.2 Oral Contraceptive Pills (OCPs)

OCPs are believed to be the preferred method for the treatment of PCOS. These regulate hormonal imbalance,acne, and hirsutism (Ehrmann et al., 2005). These drugs are considered safer than other treatments because of less chances of the endometrial cancer posed by the drugs. These pills contain a mixture of estrogen and progesterone that raises SHBG, which lowers FSH and LH, which results in lower free testosterone and decreased production of ovarian androgen (Moghetti & Toscano, 2006). Therefore, low dosage of progesterone is being advised in the OCPs. However, some side- effects of these drugs are also observed, such as impaired metabolism of glucose, IR, hyperglycemia, and T2DM.

2.10.3 Laparoscopic Ovarian Drilling (LOD)

In 1984, LOD was utilized for ovulation after ovarian wedge resection surgery was unsuccessful. LOD enhances ovarian androgen production, reduces insulin resistance, and raises SHBG levels (Farquhar et al., 2012). It is successful in 84% of patients. The levels of serum anti-mullerian hormone is used as an evaluated tool for PCOS women being treated with LOD (Amer et al., 2009). However, more research is needed to further evaluate the effectiveness of LOD.

2.10.4 Assisted Reproductive Technology (ART)

ART therapy is employed for treatment of infertility occurred in PCOS. Its procedure includes exogenous gonadotropin, which is used to stimulate ovaries and cause them to release many follicles. However in some patients, exogenous gonadotropin results in ovarian hyperstimulation syndrome (OHSS) (Tummon et al., 2005). In vitro maturation (IVM) is utilized because of the utilization of this therapy technique. Numerous research

has been done to compare the effectiveness of ART with conventional IVF methods. Results from IVM-IVF and standard IVF are comparable (Shalom-Paz et al., 2012).

2.11 Future Perspectives in PCOS Treatment

COPs are currently employed as the first-line therapy for PCOS. These include a variety of ovulation-inducing medications. The drug combinations are used to address the underlying related pathology of PCOS and raise the likelihood of conception in PCOS patients (Legro et al., 2013). However, the adverse reactions of these medications lead to a illness, such as cardiovascular pathology, T2DM, and depression (Khan et al., 2019). To lower the side effects interventional techniques were used, which are IVM fertilization, IVF, and laparoscopic techniques, but secondary disorders may also arise by using these procedures (Melo et al., 2015).

Given that PCOS has a complex pathophysiology and is difficult to treat, it is now recommended that these patients be cared for by a team of endocrinologists, doctor, gynecologist, and medicine specialist.

Chapter 3

Materials and Methods

Materials and Methods

3.1 Construction of Animal Model

To induce PCOS through constant light exposure the 6 weeks old female Wistar rats of average weight 200-220g were purchased from Animal House, ASAB. Before performing a trial, all the experiment protocols were examined and approved by the ASAB Animal Research Facility, along with IRB approval letter obtained from the Ethical Review Committee Meeting. First rats were divided into the two study groups housed in Animal House with a time difference of about a month between them. All the rats were kept at temperature of 21˚C ±2°C and humidity of 65%, along with food and water provided regularly. The two study groups were:

GROUP 1: The first group consisted of 16 female rats (age: 6 weeks, average weight: 200-220) with 4 rats per cage, which then further equally divided into two subgroups (Light/ Light and Light/Dark) with 6 per subgroup. The L/L sub-group has an induction period of 24 hours, 12/ 12 hours light, for 16 weeks (4months), while the L/D group has an induction period of 12hrs, 12 hours light/12 hours darkness, for 16 weeks (4 months). The rats of the L/L group were placed in a custom designed cage comprising of fluorescent lamp with 240W light bulbs fixed to the switch board.

GROUP 2: The second group comprised of 12 female rats (age: 6 weeks, average weight: 200-220g) with 3 rats per cage which then further equally divided into two subgroups, (Light/ Light and Light/ Dark) with 6 per subgroup. The L/L sub-group has an induction period of 24 hours, 12-12 hours light, for 8 weeks (2months), while the L/D group has an induction period of 12hrs,12 hours light/ 12 hours darkness, for 8weeks (2 months). The

rats of L/L group were placed in a custom designed cage comprising of fluorescent lamp with 600W light bulbs fixed to the switch board.

3.2 Behavioral Analysis

At the end of the PCOS induction period and before the dissection of rats, the rats were monitored for their changed behavior activity, such as memory loss and stress caused by PCOS by exposure to constant light. For behavioral analysis several experiments were performed, which are mentioned below.

3.2.1 Morris Water Maze Test

The purpose of this experiment is to test anxiety, depressive behavior, and exploratory activity of compromised rats. First, the animal was trained for 5 consecutive days to find the hidden platform in the water tank. To train the animals, five trials were conducted each day. During each of these trails, the animals were released in the water tank from different directions with an inter-trial interval of 10 mins given to each animal before each trial. The duration of each trail was 90s, during which the rats were allowed to freely explore the tank and find the platform and if animals were unable to find the platform after the 90s, they were manually placed on it for an additional 20 seconds or if they were able to find the platform within 90s, and sat on it for at least 5seconds, the time was recorded, and trial was considered over. After 5 days of training, a single probe trial was performed on the 6th day, where the platform was removed and rats were allowed to swim in the tank, trying to find the safety platform for 90 seconds. By using a video camera, the trial was recorded for video analysis, later. Using the video, the rat's number of entries into the target quadrant was recorded, along with the time spent in the target quadrant and the number of platform crossing.

Table 1 Summary of trials performed in Morris Water Maze Test

Direction of Trials for first 5 days in Morris Water Maze Test					
Days	**Trial 1**	**Trial 2**	**Trial 3**	**Trail 4**	**Trial 5**
1	West	South	North	East	South
2	North	West	East	West	South
3	North	East	West	South	North
4	West	South	West	East	North
5	West	North	East	East	South
6 Single **Trial Without Platform** **Release Direction: West**					

3.2.2 Elevated Plus Maze Test

The purpose of this experiment is to test anxiety, depressive behavior, and exploratory activity for about 5 minutes. Its setup included elevated plus maze table and video recording equipment to record the rats' behavior. The rat was put in the center of the platform with head facing open/close arms and let it explore for 5 minutes with the direction of the head during release must be kept the same in all experiments.

3.3 Blood and Tissue Sampling

All the rats were sacrificed at the end of the induction period given accordingly. The rats were anesthetized with chloroform for blood collection and dissected using a dissection box acquired from the medical institution in Islamabad. The rat was placed on the dissection plate and pinned down; an incision was done in the middle of the abdomen.

Through this incision, the animal's thoracic chamber was exposed, and blood was quickly taken from the heart by injection. Blood obtained by cardiac puncture was kept at 0℃ for 3 hours in red EDTA vials (Ethylenediaminetetraacetic acid). To measure hormone concentration, serum was also obtained by centrifuging blood at 4000rpm for 5 minutes. For histological investigation, the ovaries from group 1 were removed while kidney and liver were also collected from group 2, which were then washed with normal saline, and kept in 10% formalin solution for up to 24 hours.

3.4 Biochemical Analysis

To check the concentration of hormones affected by environmental stress (constant light), the serum concentrations of T, LH, and FSH from group 1 and serum concentrations of T, LH, FSH, AMH, cortisol, and insulin hormone from group 2 were analyzed using a biochemical analyzer. The serum biochemistry kits were used for the hormone profiling by the Diagnostic Lab, Atta-Ur-Rahman School of Applied Biosciences.

3.5 Histopathological Examination

Histopathological analysis of ovaries, liver, and kidney was done to identify the therapeutic effect of PCOS in rat models. For this purpose, histopathology of all studied groups was performed, to compare the histopathology of normal and PCOS female rats. To perform histopathology, the following methodology was adopted.

i. Dissection of female rats were done, and their ovaries, liver, and kidney were isolated.
ii. Dissected organs were then stored in 10% formalin for further examination at-80°C.

iii. Samples were dipped in the solution mixture of isopropanol and xylene at 50:50 ratio for two hours.

iv. Samples were kept in the solution mixture of isopropanol and xylene at 30:70 ratio for next two hours.

v. Samples were then immersed in 100 percent solution of Xylene for another two hours.

vi. Samples were loaded into the cast to pour the liquid paraffin.

vii. Samples were dried and sectioned by using microtome.

viii. For staining purposes, section ribbon was fixed on glass slide through cold and hot water bath cycles.

ix. Hematoxylin and Eosin (H&E) stains were used for staining purposes, followed by sealing of slide with optical aid oil and cover slip.

x. Final slides were visualized under a compound microscope at magnifying powers of 10X and 40X.

Chapter 4

Results

4.1 Behavioral Analysis

4.1.1 Morris Water Maze Test; Duration in the Target Quadrant

An experiment was conducted to evaluate reference memory. The amount of time the rat spent exploring the previously hidden platform was calculated based on how much time it spent in the target quadrant. Figure 7 shows that rats from L/D group spent more time in the target quadrant having a mean value of 0.74, while rats from L/L group spent less time in the target quadrant having a mean value of 0.4, depicting memory loss due to stress condition. The bar graph also shows a significant difference of ***P<0.0001 between the two groups. Similarly, figure 8 shows that L/D rats spent more time in target quadrant having a mean value of 0.7, while L/L rats spent less time in the target quadrant having a mean value of 0.44. The bar graph also shows a significant difference of ***P<0.0007. In addition, it has been observed that by changing the intensity of light from low to high i.e., under greater stress conditions, greater behavioral changes were observed in compromised rats.

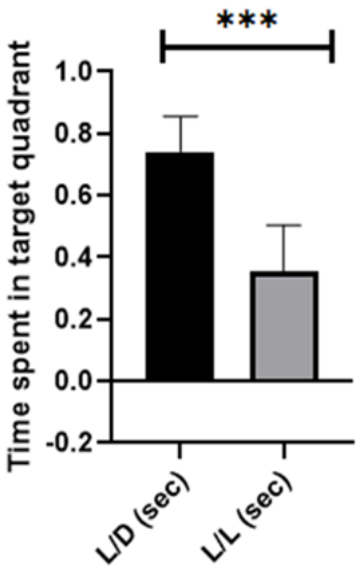


Figure 7 Bar graph representing the light induction causing PCOS on time (sec) spent in target quadrant by subjects in Morris' water maze test. L/L rats spent less time in the target quadrant than L/D group. Unpaired t-test was applied to analyze the data using GraphPad ***P<0.0001.

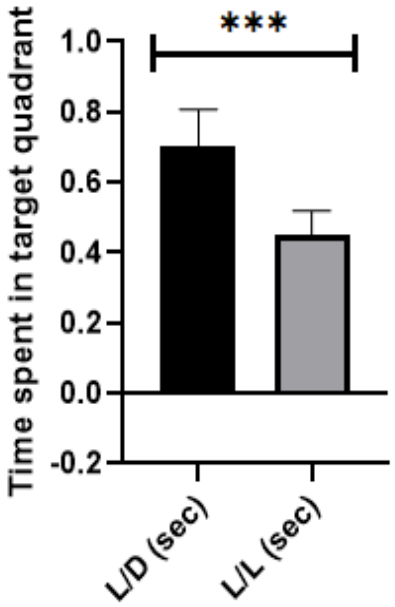


Figure 8 Bar graph representing the light induction causing PCOS on time (sec) spent in target quadrant by subjects in Morris' water maze test. L/L rats spent less time in the target quadrant than L/D group. Unpaired t-test was applied to analyze the data using GraphPad ***P<0.0007.

4.1.2 Number of Entries in the Target Quadrant in the Morris Water Maze Test

The experiment was run to evaluate anxiety, stress, and reference memory. Figure 9 shows that previously trained rats from L/D group made greater number of entries in the target quadrant having a mean value of 7.3, while rats from L/L group made lesser entries in the target quadrant having a mean value of 3.4, depicting memory loss due to stress condition. The bar graph also shows a significant difference of ***P <0.0001 between the two groups. Also, figure 10 shows that L/D rats made greater number of entries having a mean value of 8.3, while L/L rats made lesser entries in the target quadrant having a mean value of 2.67

The bar graph also shows a significant difference of ****P <0.0001. In addition, it has been observed that by changing the intensity of light from low to high i.e., under greater stress conditions, greater behavioral changes were observed in compromised rats.

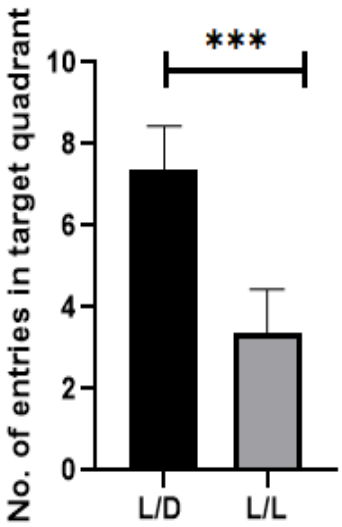


Figure 9 Bar graph depicting the effect of light- induced PCOS on number of entries made in the target quadrant by the subjects in Morris' Water Maze Test. L/L rats made significantly less entries in the target quadrant than L/D group. An unpaired t-test was applied to analyze the data using GraphPad ***P<0.0001.

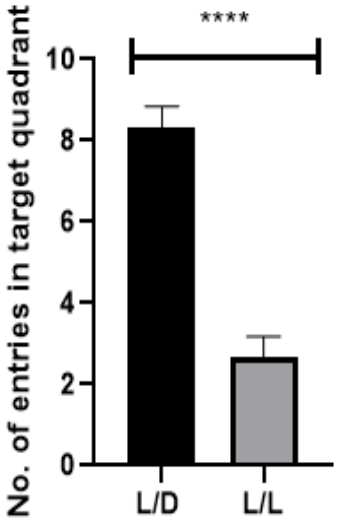


Figure 10 Bar graph depicting the effect of light- induced PCOS on number of entries made in the target quadrant by the subjects in Morris' Water Maze Test. L/L rats made significantly less entries in the target quadrant than L/D group. An unpaired t-test was applied to analyze the data using GraphPad ****P<0.0001.

4.1.3 Morris Water Maze Test; Number of Times Platform Crossing

The last experiment performed in Morris Water Maze test was the platform crossing by rats for the assessment of reference memory, stress, and anxiety. Figure 11 shows that

previously trained rats from L/D group crossed the platform more often having a mean value of 5.00, as compared to the rats from L/L group that crossed the assigned platform less frequently having a mean value of 2.75, depicting memory loss due to stress condition. The bar graph also shows a significant difference of **P <0.0063 between the two groups. Also, figure 12 shows that previously trained rats from L/D group crossed the platform more often having a mean value of 5.67, as compared to the rats from L/L group that crossed the assigned platform less frequently having a mean value of 3.33The bar graph also shows a significant difference of ***P <0.0001. In addition, it has been observed that by changing the intensity of light from low to high i.e., under greater stress conditions, greater behavioral changes were observed in compromised rats.

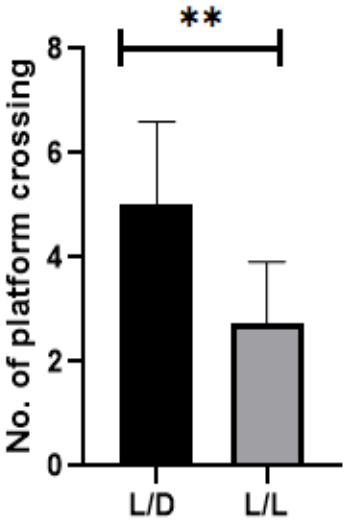


Figure 11 Bar graph depicting the effect of light- induced PCOS on the platform crossing made by the subjects in Morris Water Maze Test. L/L group made significantly less crossing over the platform compared to L/D group. A P value of **P<0.0063.

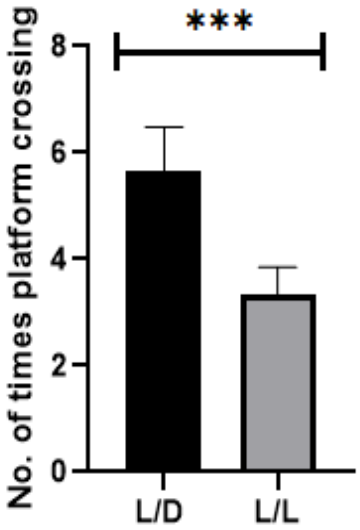


Figure 12 Bar graph depicting the effect of light- induced PCOS on the platform crossing made by the subjects in Morris Water Maze Test. L/L group made significantly less crossing over the platform compared to L/D group. A significant difference of ***P<0.0001 has been observed.

4.1.2 Elevated Maze Test; Number of Entries in Open Arm

The test is performed to analyze anxiety and depression in animal models. Figure 13 shows that rats from L/D group moved freely and made greater number of entries in an open arm having a mean value of 2.37 and rats from L/L group made much lesser number of entries in an open arm having a mean value of 1.00, depicting stress and anxiety. The bar graph also shows a significant difference of ***P<0.0001 between the two groups. Similarly, figure 14 shows that rats from L/D group moved freely and made greater number of entries in an open arm having a mean value of 3.5 and rats from L/L group made much lesser number of entries in an open arm having a mean value of 0.67. The bar graph also shows a

significant difference of **** P<0.0001. In addition, it has been observed that by changing the intensity of light from low to high i.e., under greater stress conditions, greater behavioral changes were observed in compromised rats.

4.1.2 Elevated Maze Test; Number of Entries in Open Arm

The test is performed to analyze anxiety and depression in animal models. Figure 13 shows that rats from L/D group moved freely and made greater number of entries in an open arm having a mean value of 2.37 and rats from L/L group made much lesser number of entries in an open arm having a mean value of 1.00, depicting stress and anxiety. The bar graph also shows a significant difference of ***P<0.0001 between the two groups. Similarly, figure 14 shows that rats from L/D group moved freely and made greater number of entries in an open arm having a mean value of 3.5 and rats from L/L group made much lesser number of entries in an open arm having a mean value of 0.67. The bar graph also shows a significant difference of **** P<0.0001. In addition, it has been observed that by changing the intensity of light from low to high i.e., under greater stress conditions, greater behavioral changes were observed in compromised rats.

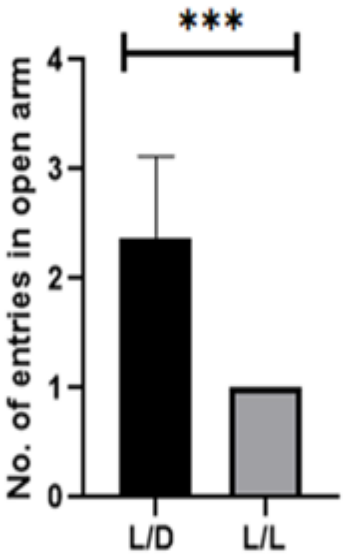


Figure 13 Bar graph depicting the effect of light- induced PCOS on the number of entries in an open arm in Elevated Plus Maze Test. L/L group made insignificantly lesser number of entries in an open arm than L/D group. Unpaired t-test was applied to analyze the data using GraphPad Prism ***P<0.0001.

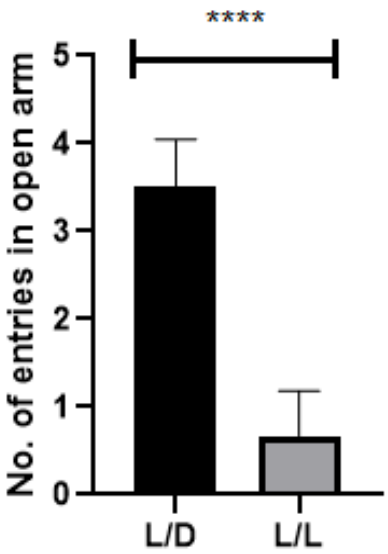


Figure 14 Bar graph depicting the effect of light- induced PCOS on the number of entries in an open arm in Elevated Plus Maze Test. L/L group made insignificantly lesser number of entries in an open arm than L/D group. Unpaired t-test was applied to analyze the data using GraphPad Prism ***P<0.0001.

4.1.3 Elevated Maze Test; Time Spent in Closed Arms

The test is performed to analyze anxiety and depression in animal models. Figure 15 shows that rats from L/D group spent less time in closed arms and moved freely having a mean value of 4.58 and rats from L/L group spent more time in closed arms arm having a mean value 4.89 of, depicting stress and anxiety. The bar graph also shows a significant difference of **P <0.003 between the two groups. Similarly, figure 16 shows that rats from L/D group spent less time in closed arms and moved freely having a mean value of 3.26

and rats from L/L group spent more time in closed arms arm having a mean value of 4.84. The bar graph also shows a significant difference of ****P<0.0001. In addition, it has been observed that by changing the intensity of light from low to high i.e., under greater stress conditions, greater behavioral changes were observed in compromised rats.

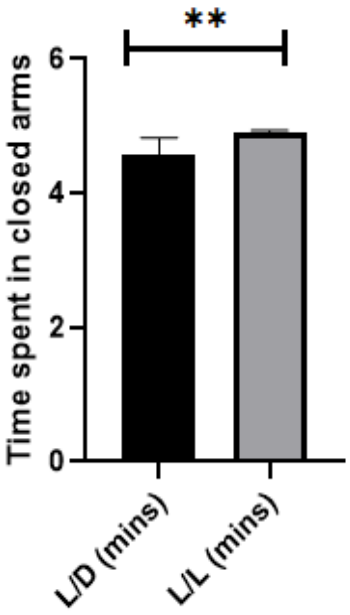


Figure 15 Bar graph depicting the effect of light- induced PCOS on time spent in close arms in Elevated Plus Maze Test. L/L group spent more time in close arm than L/D group. Unpaired t- test was applied to analyze the data using GraphPad Prism **P<0.003.

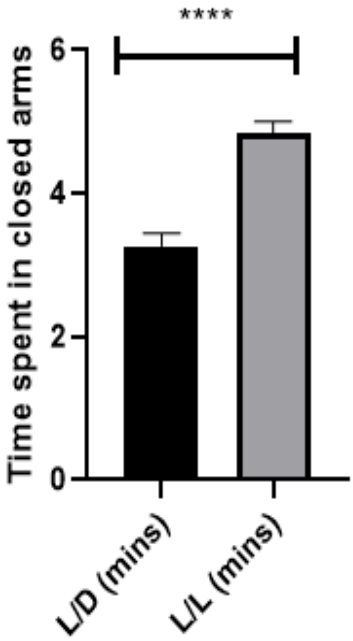


Figure 16 Bar graph depicting the effect of light- induced PCOS on time spent in close arms in Elevated Plus Maze Test. L/L group spent more time in close arm than L/D group. Unpaired t- test was applied to analyze the data using GraphPad Prism ****P<0.0001.

4.2 Hormone Testing and Clinical Profiling

To explore the effect of continuous light at different conditions such (as mentioned in a previous section) on the endocrine system, serum concentrations of hormones, including

glycoproteins, gonadotropin-releasing hormones, stress hormone, and insulin were measured. The serum concentrations of T, LH, FSH for group A, along with these hormones, insulin, AMH, and cortisol for group B were measured and compared between two groups, light-dark and light-light. An overall increase trend in the hormone levels were observed in L/L group, which is the disease group. All the results were separately mentioned below in the form of bar graphs.

4.2.1 Serum Concentration of Testosterone

The serum concentration of T was measured and compared in both groups by using ELISA kits, respectively. By using Graph Pad Prism, the unpaired t- test was applied to the data collected, and means values were compared accordingly. Figure 17 shows the serum concentration of hormone T with mean values of 1.15 for L/D rats and 1.76 for L/L rats of group A with a significance of *P <0.12 and figure 18 shows the serum concentration of hormone T with mean values of 5.92 for L/D rats and 7.61 for L/L rats of group B with a significance of ***P <0.0006. Hence, an increase trend in hormone concentration was observed in both the disease groups. Moreover, a three- fold increase in the concentration of hormones between the two disease groups has been observed by changing the intensity of light from low to high. The graphs are shown on the next page.

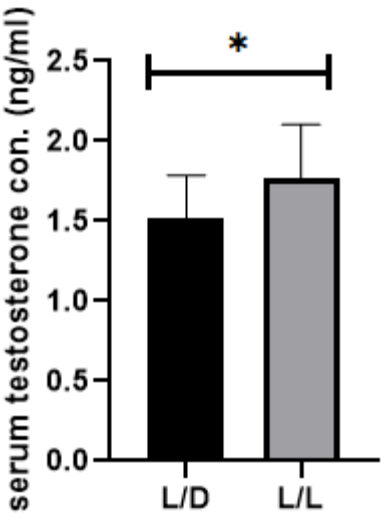


Figure 17 Bar graph shows serum concentration of testosterone in female rats kept under light- dark cycle (healthy) and light- light cycle (continuous light- induced PCOS). The L/L group reported higher levels of t than the L/D group, and a significant difference between the two groups was found.

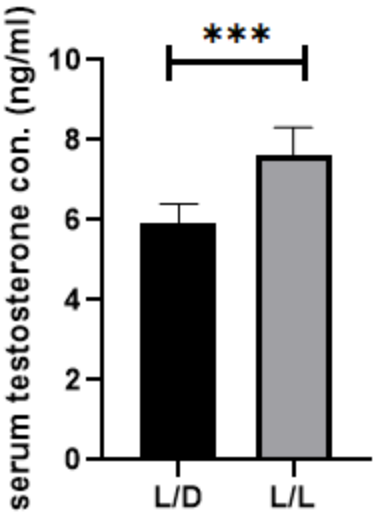


Figure 18 Bar graph shows serum concentration of testosterone in female rats kept under light- dark cycle (healthy) and light- light cycle (continuous light- induced PCOS). The L/L group reported higher levels of t than the L/D group, with a three-star significant difference between the two groups being noted.

4.2.2 Serum Concentration of LH

The serum concentration of LH was measured and compared in both groups by using ELISA kits, respectively. By using Graph Pad Prism, the unpaired t- test was applied to the data collected, and means values were compared accordingly. Figure 19 shows the serum concentration of LH with mean values of 5.02 for L/D rats and 7.62 for L/L rats of group A with a significance of ***P <0.0001 and figure 20 shows the serum concentration of LH with mean values of 0.03 for L/D rats and 0.12 for L/L rats of group B with a significance

of ***P <0.0001. Hence, an increase trend in the hormone concentration was observed in both the disease groups. Although both groups have three-star significance, it has been observed that by changing the intensity of light the concentration of hormones changes slightly with no significant difference.

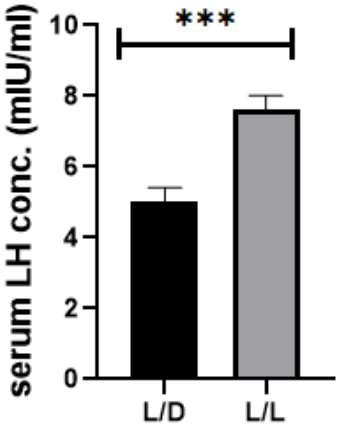


Figure 19 Bar graph shows serum concentration of luteinizing hormone in female rats kept under light- dark cycle (healthy) and light- light cycle (continuous light- induced PCOS). When comparing the L/L group to the L/D group, it was found that the LH levels were higher in the L/L group, with a three-star significant difference between the two groups.

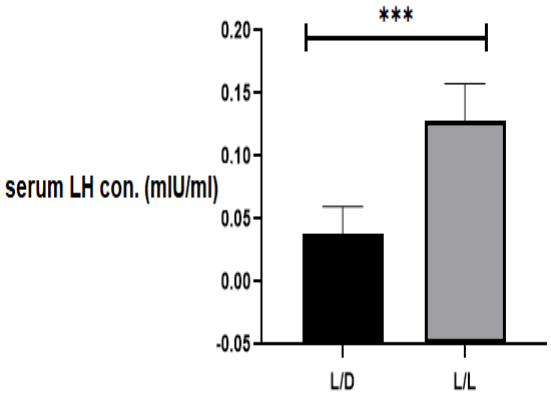


Figure 20 Bar graph shows serum concentration of luteinizing hormone in female rats kept under light- dark cycle (healthy) and light- light cycle (continuous light- induced PCOS). In comparison to the L/D group, the L/L group reported higher levels of LH, with a three-star significant difference between the two groups.

4.2.3 Serum Concentration of FSH

The serum concentration of FSH was measured and compared in both groups by using ELISA kits, respectively. By using Graph Pad Prism, the unpaired t- test was applied to

the data collected, and means values were compared accordingly. Figure 21 shows the serum concentration of FSH with mean values of 6.56 for L/D rats and 6.52 for L/L rats of group A with a significance of ***P <0.0004, while figure 22 shows the serum concentration of FSH with mean values of 0.13 for L/D rats and 0.12 for L/L rats of group B with a significance of *P <0.0001. Hence, a decrease trend in the hormone concentration was observed in both the disease groups. Moreover, with changing the intensity of light from low to high the change in the concentration of hormones between the two disease groups was also notified with group B showing a three- fold increase in hormone levels.

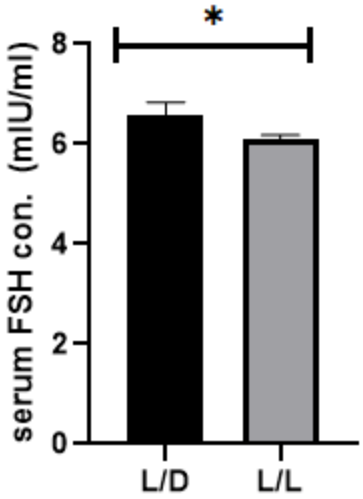


Figure 21 Bar graph shows serum concentration of follicle stimulating hormone in female rats kept under light-dark cycle (healthy) and light- light cycle (continuous light- induced PCOS). When compared to the L/L group, the FSH levels in the L/D group were higher, and a three-star significance test revealed.

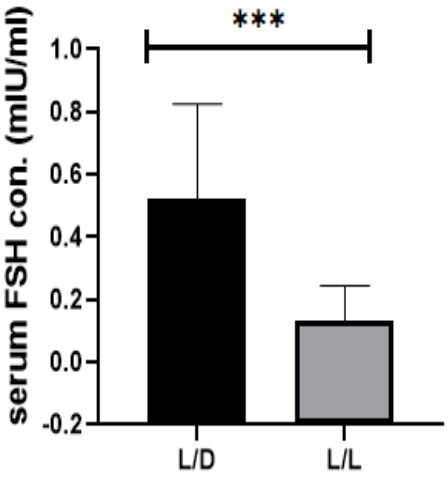


Figure 22 Bar graph shows serum concentration of follicle- stimulating hormone in female rats kept under light-dark cycle (healthy) and light- light cycle (continuous light- induced PCOS). When compared to the L/L group, the L/D group was shown to have higher levels of FSH, with a one-star difference between the two groups being noted.

4.2.4 Serum Concentration of Insulin Hormone

Serum concentrations of insulin have been measured and compared of both groups. Figure 23 shows that L/D rats have normal range of insulin in their blood, along with the mean value of 1.92, while insulin concentration has increased in L/L rats depicting the mean value of 5.32 showed insulin intolerance. Further, unpaired t- test was applied and graph was plotted showing the significance of ***P<0.0002 by using the Graph Pad Prism.

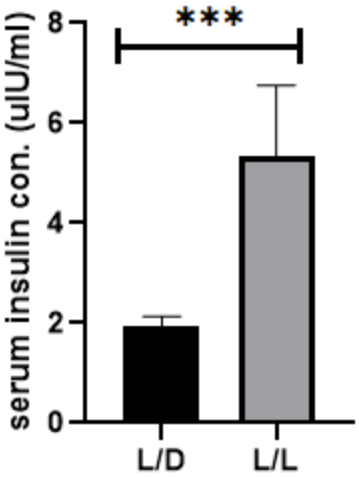


Figure 23 Bar graph shows serum concentration of insulin in female rats kept under light- dark cycle (healthy) and light- light cycle (continuous light- induced PCOS). When comparing the L/L group to the L/D group, elevated insulin levels were found, and the three-star significance test revealed a significant difference between the two groups, showing insulin intolerance.

4.2.5 Serum Concentration of AMH

Serum AMH concentrations were measured and compared in both groups. Figure 24 shows L/D rats have less concentration of AMH having a mean value of 2.58, and L/L rats have high concentration of AMH having a mean value of 2.94. Additionally, an unpaired t-test was used with GraphPad Prism, and a graph was created to show the significant difference between the two groups (*P0.01).

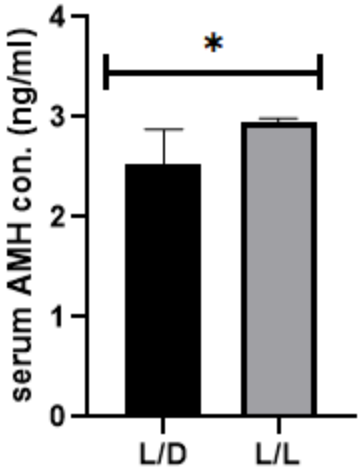


Figure 24 Bar graph shows serum concentration of anti- mullerian hormone in female rats kept under light- dark cycle (healthy) and light- light cycle (continuous light- induced PCOS). The high levels of AMH were reported in experimental group as compared to the healthy control with the slight difference between the two groups being observed with one-star significance.

4.2.6 Serum Concentration of Cortisol Level

Serum concentration of cortisol was measured and compared by ELISA hormone testing. Figure 15 shows that L/D rats have less concentration of cortisol having a mean value of 25.9 and L/L rats have higher concentration of cortisol in their blood having a mean value of 32.4. The unpaired t- test was applied, plotted graph showed significance of *P<0.039 by using Graph Pad Prism. The graph is plotted on the next page.

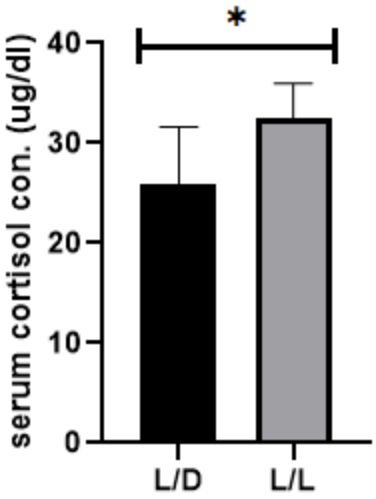


Figure 25 Bar graph shows serum concentration of cortisol in female rats kept under light- dark cycle (healthy) and light- light cycle (continuous light- induced PCOS). The high levels of cortisol were reported in experimental group than healthy control with the slight difference between the two groups being observed with one-star significance.

4.3 Histopathological Examination of Tissues

To determine the effect of constant light, as well as the correlation between circadian rhythm and PCOS, and its effect on reproduction and metabolism histopathological examination of ovaries, liver, and kidney was performed using Hematoxylin and Eosin (H& E) stain in order to understand prognosis of PCOS. In histopathology staining scheme, the shape of cells, their structure and the arrangement of cells, and arrangement of cells in the tissue was determined. All the steps of histopathological staining: Dehydration, microtome cutting, staining and microscopic analysis, were performed in the ASAB lab.

4.3.1 Histopathology of Ovary

Rat ovarian tissues are analyzed under a compound microscope at resolution of 10X and 40X. L/L rat's ovaries showed greater number of follicles compared to L/D rats in both the groups (A& B). The overall number of large antral-follicles, tiny antral-follicles, and follicular fluid-containing oocytes, as well as large fluid-filled cysts with an attenuated

granulosa cell layer are known as atretic cyst-like follicles are increased in L/L rats of group B, along with the ruptured follicles, inflamed ovary, and cysts.

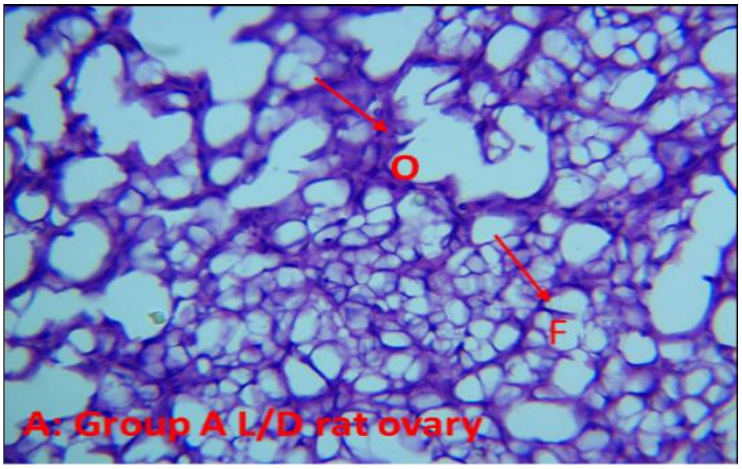


Figure 26 A: L/D rats (healthy group) show ovary with follicle and follicle growth.

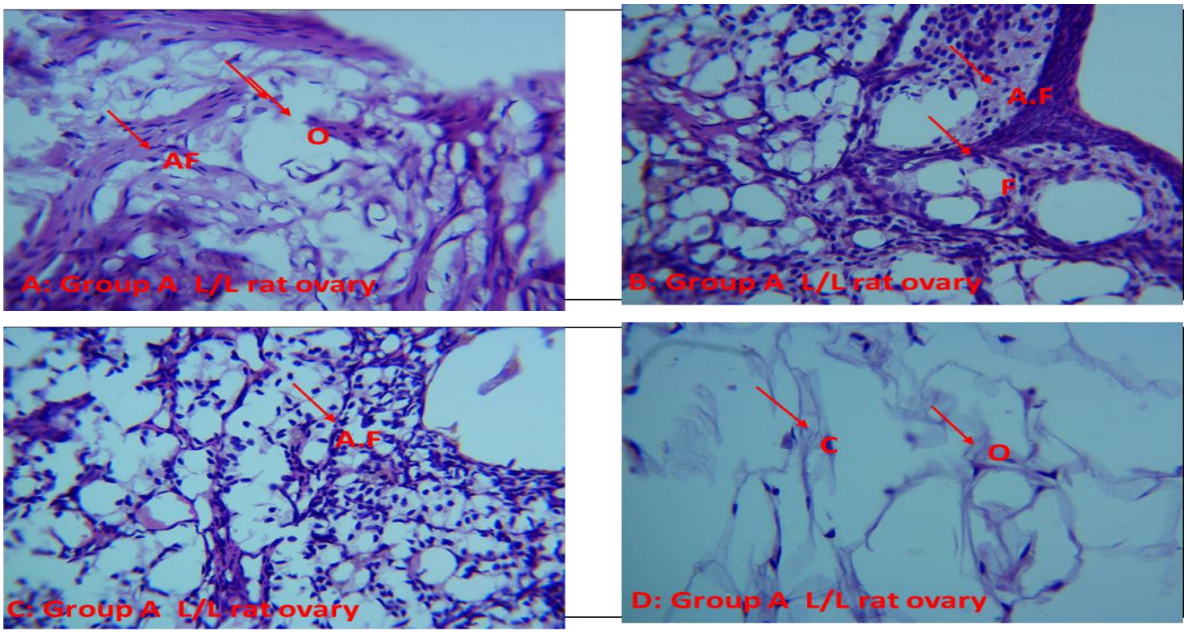


Figure 27 A: L/L rats show inflamed ovary (O) with small antral- follicles (AF). B: L/L rats show ruptured follicles (F) with small antral- follicles (AF). C: L/L rats show ovaries with small- antral follicle growth (AF). D: L/L rats show few cysts (C), along with antral follicles (AF) in ovary.

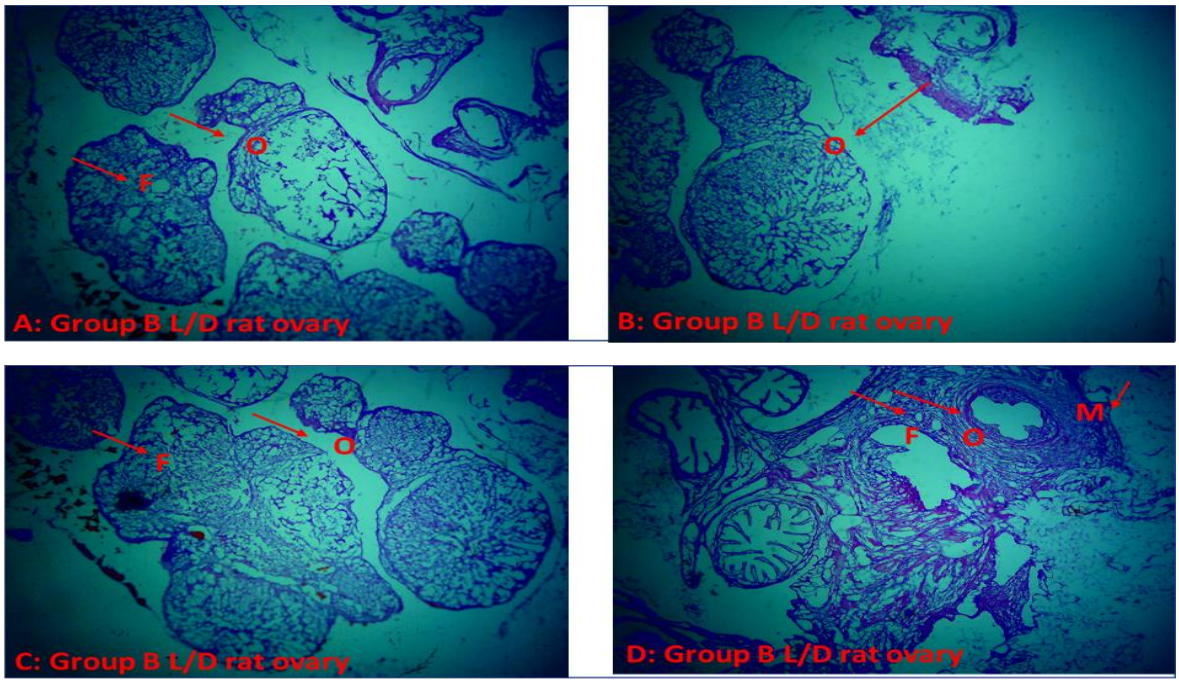


Figure 28 A: Healthy rats show well- structured ovaries (O) and follicles (F) with follicle growth. B: A healthy ovary (O) has been demonstrated. C: Light- dark cycle group (L/D rats) were observed with follicle growth (F) in ovaries. D: L/D rats have structured membrane- lining (M) around ovaries, along with follicles (F) in them.

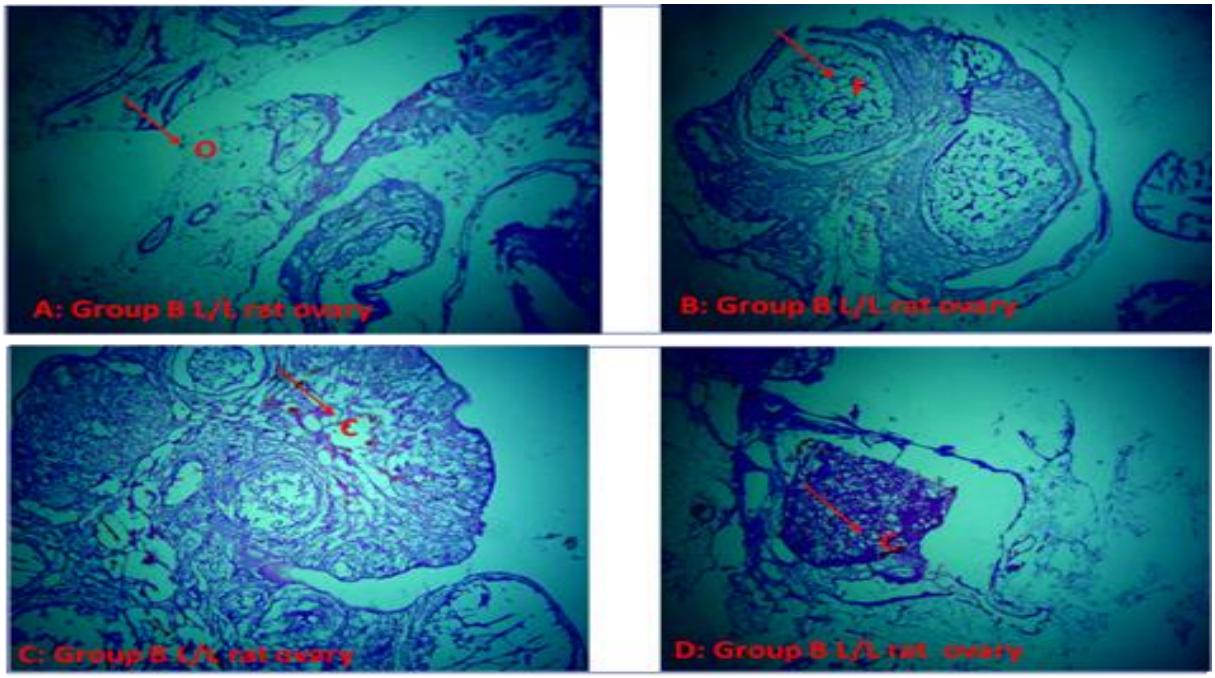


Figure 29 A: Continuous light- induced PCOS rats show inflamed ovaries (O). B: L/L group show ruptured follicles (F) due to the light- induced POCS. C: Disease rats were observed with several cysts (C) in their ovaries. D: L/ L rats were observed with antral follicles or cysts (C) in their ovaries.

4.3.2 Histopathology of Liver

Group B rats were further examined for liver complications occurred because of continuous light on female rats for the possibility of PCOS. The liver does not have a particular microscopic appearance in PCOS-affected rats. However, degenerated hepatocytes with swollen boundaries were reported in the L/L rat's liver, whereas L/D rats' liver was seen with proper hepatocytes with clear boundaries between them.

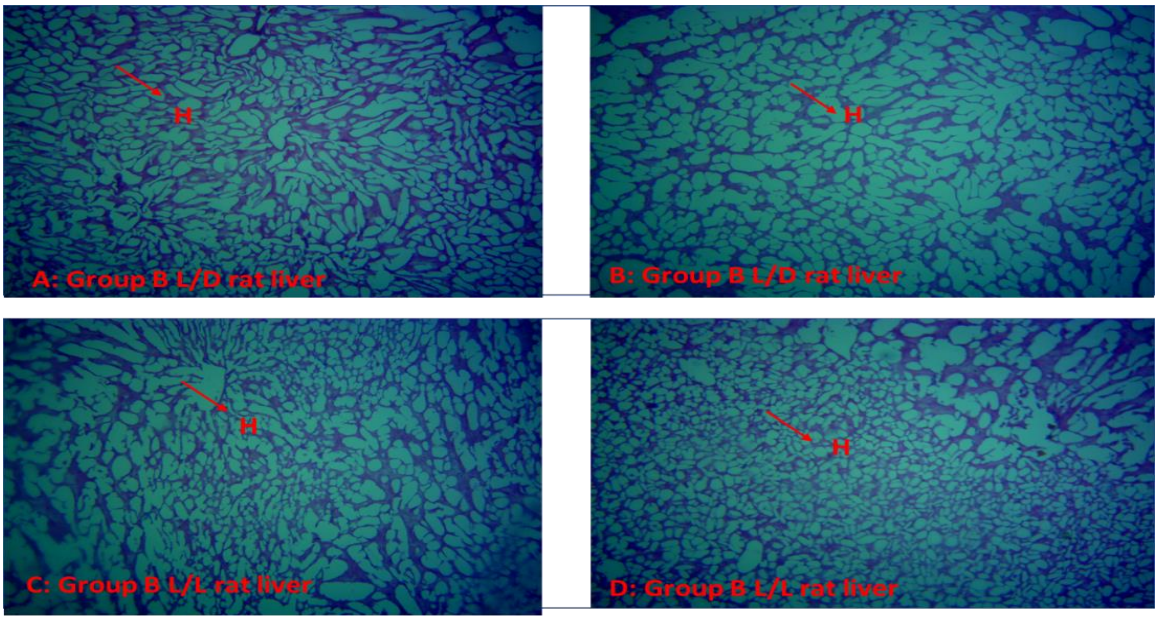


Figure 30 A: L/D rats (healthy group) show liver with hepatocytes (H). B: L/D rats show hepatocytes with cells (H). C: L/L rats (continuous light- induced PCOS) show liver with degenerated hepatocytes (H). D: L/L rats show degenerated hepatocytes with congested cells and boundaries.

4.3.3 Histopathology of Kidney

Group B rats were also examined for kidney disorders that may occur because of continuous light on rats for the possibility of PCOS. Like liver, kidney does not have a particular microscopic appearance in PCOS- affected rats. However, the kidney of L/L rats

was reported with reduced glomerular cellular- density, along with degenerated lining of cells, and few peritubular capillaries were dilated and congested.

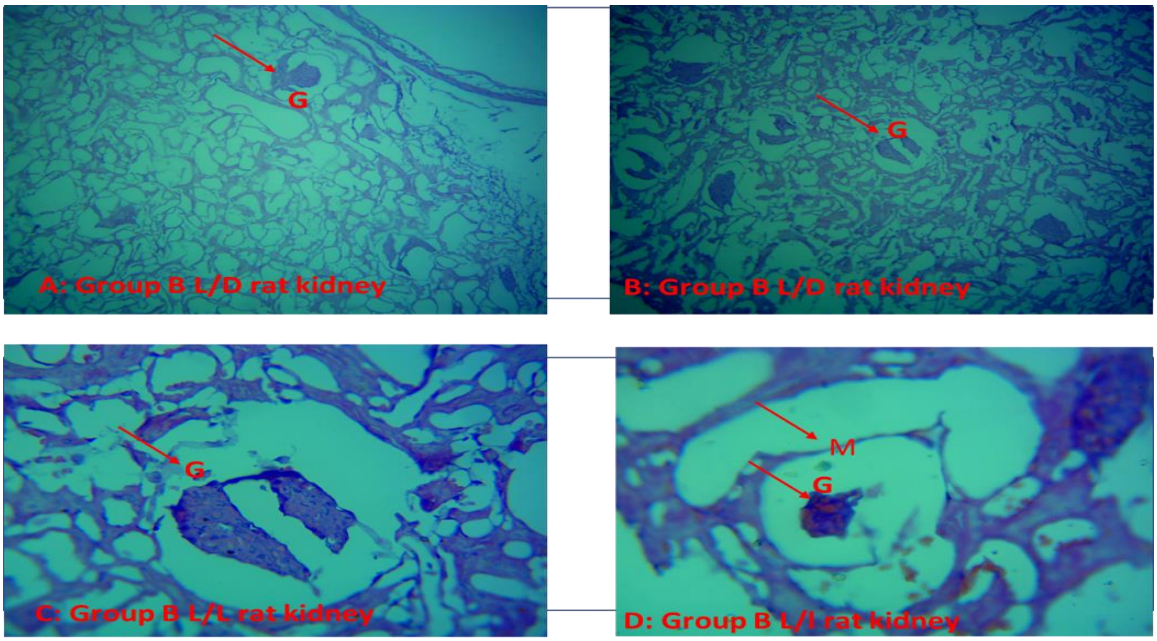


Figure 31 A: L/D rats show kidney with proper cellular structure and glomerulus (G). B: L/D rats show kidney glomerulus with proper cell lining. C: L/L rats show kidney with degenerated lining cells and degenerated glomerulus. D: L/L rats show decreased glomerulus density, along with dilated and congested capillaries.

Chapter 5

Discussion

5 Discussion

PCOS is caused by an abnormal interaction of various genetic, environmental, and behavioral variables. The increased androgenic secretion is a result of enzymatic overactivity, which is involved in the steroid synthesis process (Saadia, 2020b). Many studies have reported that the LH to FSH ratio is the proportion of luteinizing hormone and follicle-stimulating hormone levels in the blood. In healthy women, this ratio (LH: FSH) usually lies between 1 and 2, while in women with PCOS, LH levels are usually elevated, and the ratio can be 2:1 or 3:1, which can affect ovulation. Several studies also mentioned concerns abouts the limitation of inconsistency of this ratio because of which it cannot be used as a diagnostic criterion for PCOS. In addition, a lot of women with PCOS exhibit other symptoms of the disorder and have normal levels of LH and FSH (Li et al., 2020). The aim of our research is to induce PCOS in an animal model by exposure to constant light environment and establish PCOS- like symptoms in animal models. The current research findings are consistent with the previous research according to which, the disorder of light conditions leads to the hormone level changes in female rats, as serum testosterone levels of female rats increased in a continuous light environment. The study also discovered that female rats under a constant light had lower body weights, along with uterine and ovarian enlargement, as well as disturbed estrous cycles and polycystic ovary-like alterations, accompanied by lethargy and fur loss (Chu et al., 2020).

According to a study, unsuitable environment, including stress conditions, can cause rats to develop PCOS-like symptoms involving hormonal imbalance as well as changes at tissue levels. The study found that Continuous light exposure was discovered to cause PCOS in female rats without the requirement for medication; this is a suitable PCOS animal

study that is more in line with the condition of the disease in humans. Additionally, poor sleep hygiene or poor sleep cycle may be a significant lifestyle factor in the development of PCOS (Kang, Jia, and Shen 2015) (Kang et al. 2017). In current study, we closely focused on hyperandrogenism, insulin intolerance, and organs impairment by subjecting two groups of rats to different environmental conditions, including constant light, along with its impact of on reproductive and metabolic changes in female rats were further investigated. According to a study, the long-term change in the light regime can affect the level of sex hormones, testosterone, and estradiol in the blood plasma of rats. The study found that light at night can affect LH, FSH and Testosterone in the Wistar rats. Also, light phase exposure to LED lighting can affect hormone concentration in Sprague-Dawley rats like the previous research conducted on Light- Induced PCOS (S. Li et al., 2020). The present study demonstrates overuse of light and light pollution disrupts a natural circadian cycle, which is negatively affecting about 62% of world's population. This result analysis was consistent with the previous study (Belenkaia et al., 2019).

Many studies observed slight or no change in the levels of serum LH and FSH with only the pattern of LH: FSH ratio is affected by the constant light environment (Saadia, 2020a). According to current study, considerable change in the concentrations of LH and FSH with a significant increase in LH: FSH ratio provides clear evidence of the effect of the environmental stress causing the arrhythmic expression of circadian biological clock. It indicates serum concentration of LH increases with the significant difference of *** P <0.0001 and serum concentration of FSH decrease with significant difference of *** P <0.0001, along with an overall increase in LH: FSH ratio in the both groups. Additionally, this study also provides comparison between the two groups with reporting a three- fold

increase in the concentration of hormones due to the high light intensity used in the second group. These results are similar to the previous research findings (S. Li et al., 2020). It has also been reported by our study and previous study that light intensity has been found to increase serum LH levels which is directly correlated with the increased circulating testosterone level (Malini & George, 2018). The pathogenic prerequisites of PCOS include abnormality of the hypothalamic-pituitary-ovarian or adrenal axis along with the disruption in the GnRH secretion pattern which leads toward the relative increase in LH: FSH level, as well as an aberrant feedback mechanism that increased LH release was driven on by ovarian estrogen (Saadia 2020b).

According to current research, there is similar evidence indicating an increase in the levels of testosterone as reported by prior studies, indicating strong hyperandrogenism in research models. The serum testosterone levels in female rats increased significantly with value ***P<0.01 (Abinaya et al., 2019). It provides definite evidence that unstable lifestyle choices and poor sleeping patterns may contribute to the development of PCOS- like symptoms. This study also has various strengths, including the measurement of testosterone by biochemical testing, which shows significant difference in the levels of testosterone that might lead to the PCOS reproductive- like changes (Ashraf et al., 2019). According to research conducted in 2020, there was an unchanged serum concentration of testosterone which removed the evidence of stress and reproductive-like changes in PCOS. (Chu et al., 2020). According to experiment conducted on another group of female rats with changed light intensity of 600W and time duration of 8 weeks, serum concentration of testosterone increased three times than in the previous group of rats. Hence proved that hyperandrogenism is caused by the disturbed circadian clock. As mentioned earlier that

many studies have reported a co-relation between insulin resistance and PCOS and women suffering with PCOS are mostly likely to develop IR (Marsh et al., 2010) (Mo et al., 2022).

According to a study1, hyperandrogenism causes a series of pathophysiological changes in PCOS patients, including insulin resistance, hyperinsulinemia, dyslipidemia and an unbalanced LH/FSH ratio. These changes not only singly promote PCOS development but interact to form a vicious cycle that induces PCOS.

We have observed that rats exposed to constant light had high levels of insulin and developed IR, due to which an increase concentration of androgens in the rat's blood was measured. According to a study, the relationship between continuous light-induced hyperandrogenism and hyperinsulinemia is bidirectional. Our research findings also demonstrate that increased insulin in blood can lead to over synthesis of androgen by ovaries, which further worsen the growth and development of PCOS in our animal models. This research finding corroborates with the other studies as well (Zeng et al., 2020). According to a study, treatment with insulin for 2 days resulted in significant increases in both the basal and the maximal release of LH and FSH, as well as a 3.2- and 6.3-fold decrease in the ED50 values for GnRH in terms of LH and FSH release, respectively (Armanini et al., 2022). In our animal models induced with PCOS, the high concentration of insulin in serum was found, leading to the imbalance in estrogen and androgen production (Aziz et al., 2023).

Research has also shown a clear interlink between high levels of AMH and PCOS (Teede et al., 2019). Studies show that women with PCOS can have an increased AMH level by up to four times the "normal" value for age. Research has also found that ovaries in women

with PCOS who don't get their periods secrete AMH up to 75 times higher than controls. AMH is elevated and associated with the multiple metabolic/endocrine and reproductive disturbances associated with PCOS. AMH suppresses the growth and recruitment of follicles, which causes follicular arrest. High levels of anti-Mullerian hormone (AMH) have traditionally been thought of as merely a passive byproduct of polycystic ovary syndrome (PCOS), but a new preclinical study by Weill Cornell Medicine researchers suggests that the hormone plays an active role in the disorder and may contribute to problems with ovulation and fertility (Zhang et al., 2023).

Despite a complex relationship between AMH and PCOS, few studies have recommended AMH as an essential biomarker for PCOS diagnosis. A metanalysis and systematic review of numerous studies identified that women suffering with PCOS had higher concentrations of serum AMH as compared to women without the disorder (Dewailly et al., 2020). Continuous exposure to light is crucial for causing AMH increase, and disturbance of the circadian rhythm may be essential for the pathological development of ovulation failure in PCOS. Even without the use of synthetic steroid hormones, the same increase in AMH levels was seen (Chu et al., 2020).

According to some studies, many small sacs of fluid develop along the outer edge of the ovary, which are called cysts, in women suffering from PCOS. These small fluid-filled cysts contain immature eggs called follicles. These follicles fail to regularly release eggs (Dennett & Simon, 2015). These follicles fail to regularly release eggs. However, the exact cause of PCOS is unknown, several studies show that high levels of serum AMH in PCOS are due to the greater number of immature small follicles in ovaries (Moolhuijsen & Visser, 2020). Despite an unknown mechanism by which PCOS is caused due to the high

concentration of serum AMH is unknown, we have identified that high concentration of serum AMH might be involved in the hormonal imbalance inside the body that regulates the menstrual cycle and ovulation, as reported by previous studies (Vagios et al., 2021). We have also observed elevated levels of serum AMH in PCOS because there are more small follicles and there is more secretion coming from each of them. Multiple immature follicles may form as a result of the high AMH level, preventing the ovaries from developing normally. This may result in PCOS-like symptoms, such as disturbed menstrual cycle and loss of fertility. These results are in contrast with the previous study (Ou et al., 2021). Furthermore, we have also proved that high concentrations of serum AMH can also induce production of hormones, such as androgens, as high levels of t- hormone were documented in the experimental group, which is in accordance to the previous studies (Chu et al., 2020) (Sahmay et al., 2013).

Other than these hormones, we have studied stress hormone, cortisol, and determined its serum levels in female rats that were subjected to the environmental stress of constant light.

Cortisol has been implicated as a sensitive biomarker for stress-related changes in the body, playing an essential role in metabolic homeostasis. It has been found that stress can have an impact on the cause and worsening of PCOS. Cortisol keeps the body in a state of high alert, whilst also increasing blood glucose levels, suppressing insulin levels, reducing our immune response and wound healing, and altering our appetite and mood. There are increasing reports linking the role of stress in PCOS manifestation (Singh et al., 2023).

We have also conducted behavioral analysis on rats to establish whether animals' behavior is impacted by continual light, which can be a new hallmark for PCOS. PCOS can cause

changes in behavior such as memory loss, anxiety, and depression. It can cause mood swings and irritability according to other studies (Riezzo et al., 2023). The first test performed was the Morris Water Maze Test, which showed that rats under environmental stress affected by light- induced PCOS spent less time in target quadrants showed little or no movement (Salman et al., 2019). Moreover, animal models were observed to have little or no memory of the platform's location and were unable to cross it at the specific position. In contrast, healthy rats were more active with proper memory generation in them (Tian et al., 2019). Another experiment conducted on the continuous light- induced PCOS rat models was Elevated maze test. This test also exhibited stress and memory loss behavior in disease rats (Zhang et al., 2020). The rats with the disorder showed little movement over the open arm and preferred to remain in the closed arms, while healthy rats were freely moving from one corner to another. (Guillén-Ruiz et al., 2021). The present findings suggest that rats exposed to constant light experienced high levels of stress, anxiety, and depression. Our study also found evidence that continuous light exposure affected ovarian morphology. The L/L group showed features of PCOS, including an abundance of follicles that resembled atretic cysts and a decrease in the number of CLs, which indicated oligo/anovulation. In addition, ruptured follicles, inflamed ovaries, and cysts were observed in PCOS rats. All these modifications complied with earlier reports (Kang et al., 2015) (Chu et al., 2020). Histopathological analysis of liver and kidney for rats of Group B showed that degenerated hepatocytes with swollen boundaries were observed in disease models, whereas liver of healthy models had hepatocytes with clear boarder between them (Abulfadle et al., 2022) (Akintayo et al., 2021). Similarly, kidney disease model was also affected by constant light, as disrupted glomerulus was recognized with dilated and

congested cells reported. All these findings were in align with the previous studies (Alahmadi et al., 2021). Major environmental changes cause rats to experience stress and anxiety. The response of HPA axis's to the synthesis of high cortisol is linked to sleep deprivation, which can cause partial sleep deprivation. Due to this disturbance in sleep patterns, rats underwent anxiety, depression, and distorted behavioral activities (Kang et al., 2015).

This work established the significance of disrupted circadian rhythms in the emergence of the PCOS-like symptoms, including reproductive hallmarks, and identified a potential pathophysiologic basis for PCOS, as well as circadian rhythm disruption being involved in the development of metabolic diseases and subfertility. Another study suggests that circadian rhythm disorders may be one of the causes of excess androgen in PCOS. It clarified a direction for further research into the pathologic causes of PCOS and offered a preliminary data for the clinical diagnosis in the reproductive age group of women experiencing altered circadian rhythms.

Chapter 6

Conclusion

6 Conclusion

In conclusion, the exposure of rats to constant light mimics the PCOS- like symptoms, including anxiety and depression that characterize human sleep disorders. Rats under such environmental stress appeared to have reproductive and metabolic changes. It has been proved that there were pathologically high levels of androgens in the L/L model, along with increased ratio of LH: FSH. Besides, elevated concentrations of insulin and AMH that further contribute to PCOS by increasing serum testosterone were measured. An interlink between IR and PCOS was identified as hyperinsulinemia due to glucose imbalance that was observed in the disease model. Additionally, due to environmental stress, cortisol increases in L/L animal model. Moreover, tissue damage was also observed in continuous light- induced PCOS rats. The constant light simulates PCOS in individuals and presents a more accurate actual clinical state of PCOS. The pathologic physiological phenomena depicted in this model could reveal novel PCOS causes. The data suggests hormonal imbalance in light- induced PCOS comparable to the hormonal imbalance in reproductive women suffering with PCOS.

Chapter 7

Innovative Strategies

7 Innovative Strategies for PCOS

7. 1 Lifestyle and Behavioral Strategies

International evidence-based guidelines (2018) for the evaluation and treatment of PCOS advised lifestyle therapies such as exercise, diet, and behavioral strategies for women suffering with PCOS. Earlier research using a mouse model of hyperandrogenic PCOS demonstrated that nutrition could selectively enhance certain PCOS features. It was discovered that reproductive qualities are more susceptible to the balance of dietary macronutrients than metabolic traits, indicating that the introduction of evidence-based dietary treatments would be a promising method for managing PCOS, especially for reproductive features (Rodriguez Paris et al., 2020).

The behavioral and lifestyle approach has long been regarded as the first line of PCOS treatment, particularly for overweight and obese women (Bates & Legro, 2013). Some of the first-line lifestyle therapies for PCOS-affected women have been proposed to include increased dietary changes, physical activity, and weight loss (Domecq et al., 2013) (Norman et al., 2002) (Bates & Legro, 2013). For the long-term management of the disease, LBA should also account for the changes in tobacco usage, psychosocial stress, and alcohol intake (Norman et al., 2002). LBA has been proven to have the greatest impact on PCOS women with hyperglycemia and obesity, but it has had little to no impact on ovulation problems and hyper androgenicity in non-obese women (Domecq et al., 2013). There always has been a relation between obesity and PCOS, hence preventing weight gain is necessary for the management of PCOS (Brennan et al., 2017). Evidence supports that

reduction in weight loss leads to the decrease in insulin resistance and restores fertility in PCOS women (Norman et al., 2002).

7.2 Pharmaceutical Approaches

To reduce androgen levels and relieve symptoms oral contraceptives in combination with antiandrogens remain the standard therapeutic strategy (Luque-Ramirez et al., 2018). By exerting negative feedback on the hypothalamus, they decrease ovulation while concurrently reducing ovarian androgen output (Shah et al., 2018). According to recent research, treating PCOS patients with a combination of 3 milli gram of drospirenone and 20 grams of ethinyl estradiol is effective for hormonal imbalance and lipid profile while having a high level of safety (L. Li et al., 2020). For an anovulatory PCOS females, ovulation stimulation with clomiphene citrate (CC) remains the treatment of choice. In individuals who failed CC treatment, 2.5 mg/day letrozole (an aromatase inhibitor) has been proposed as an option to stimulate ovulation.

7.3 Surgical Management

Laparoscopic ovarian drilling (LOD), which was first used in 1984, has become a highly effective and safe surgical technique for PCOS patients who are not responding to the CC medication. Along with a notable improvement in ovarian responsiveness to subsequent therapy with ovulation induction drugs, it mimics the effects of gonadotropins using pregnancy and live births omitting dangers of ovarian hyperstimulation or multiple pregnancies (Mitra et al., 2015).

Chapter 8

References

8 References

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